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ENDOCRINOLOGY

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EFFECT OF PITUITARY GROWTH SUBSTANCE ON THE DEVELOPMENT OF RATS THYROIDECTOMIZED AT BIRTH[1]

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ALTHOUGH both thyroid and pituitary play a part in the normal growth and development of the individual, the pituitary can, under certain circumstances, act without the thyroid. Evans, Simpson and Pencharz (1), also Flower and Evans (2), induced growth in rats thyroidectomized after weaning by injection of AP growth substance. Ossification of epiphyseal lines was accelerated by pituitary growth hormone in thyroidectomized guinea pigs (120–180 gm.) (3). On the other hand, Shelton, Tager and Hoyt (4) found it impossible to cause osseous advancement in a human cretin with pituitary substance alone.

In a previous experiment by the author (5) growth was not induced by two pituitary implants daily in rats completely thyroidectomized at birth, although one pituitary implant daily was sufficient to cause growth in animals possessing a thyroid remnant. In that case an interval of 3 to 4 weeks elapsed between thyroidectomy and the beginning of pituitary administration. Evans, Simpson and Pencharz (2) showed that with older rats the interval is not significant since rats thyroidectomized at 35 to 45 days could still be made to grow if pituitary growth extract injections were begun as late as 3 months after thyroid removal. They suggested, therefore, that the two pituitaries daily used by us in newborn thyroidectomized rats constituted a dose insufficient to produce growth. The purpose of the present work was to determine whether growth could be induced in rats thyroidectomized at birth with relatively large doses of growth hormone, or whether the difference in the ages of the rats at the time of thyroidectomy was a factor responsible for the difference in response.

EXPERIMENTAL PROCEDURES

Newborn rats of the Long-Evans strain were thyroparathyroidectomized according to the method described previously (5). The heavier animals in each litter were chosen for operation and the lighter as untreated controls. The controls thus represent the minimum weight and development which the experimentals could have attained had they been permitted to develop normally. At about 4 weeks of age, when the newborn thyroidectomized animals had begun the growth plateau which they

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[1] Aided by a grant from the Rockefeller Foundation administered by Dr. Philip E. Smith.

regularly reach (5, 6), injections of 3 U growth hormone[2] daily were begun. The distribution of animals is shown in table 1.

Since there was some possibility that two pituitaries daily, as previously reported (5), were an inadequate dose to produce growth in newborn thyroidectomized rats, the dosage in the present series was gauged, as far as calculations could be used as a guide, so that the amount given should be more than enough to cause growth in older thyroidectomized animals. A comparison of the dosage used in the present series with the dosage used by Evans, Simpson and Pencharz (2) is as follows. Squibb extract is standardized in units, one unit being the amount which, if injected in 10 consecutive daily doses, will result in a 10% increase in the weight of female rats (250 ± 25 gm.). Evans' growth extract (7) gives an average gain of 35 gm. in 20 days when injected in daily doses of 1/8 cc. and 55 gm. when given in 1 cc. daily doses. His extract is not

TABLE 1. DISTRIBUTION OF ANIMALS

	Controls	Newborn thyro-parathyroidectomized						
				3 U Growth hormone daily after weaning time				
					No growth		Growth	
		No. operated	Survivors	No. injected	No thyroid remnant	Thyroid remnant	No thyroid remnant	Thyroid remnant
Male	24	69	29	21	8	3[2]	0	10
Female	18	59	21	14	6	2[2]	1[3]	5
Totals	42	128	50[1]	35	19		16	

[1] Fifteen of these animals were discarded since they did not remain in a growth plateau and were therefore obviously incompletely thyroidectomized.

[2] These animals were not healthy. They lived only about a week after injections were begun.

[3] No thyroid remnant was found in the tracheal region or thymus, but the animal's behavior indicated that some aberrant thyroid tissue was probably present.

measured in units. Since a 55 gm. increase in 20 days is approximately twice the 25 gm. increase caused by the Squibb extract in 10 days (Evans' growth curves appear to be a straight line), it would seem that 1 cc. of Evans' extract contains the equivalent growth substance of 1 U of the Squibb preparation. Evans, Simpson and Pencharz injected 1 cc. of growth hormone daily into rats between 100 and 200 gm. in weight. In the present experiment the dosage was 3 U (Squibb) per 30 gm. rat, daily, or approximately 9 times the amount per unit weight used by Evans, Simpson and Pencharz. It seems reasonable that such a dose would be adequate if the newborn thyroidectomized rats possessed any capacity for response at all.

The animals were weighed every other day until injections were begun after which time daily weights were recorded. Only those rats which ceased growing after 23 days were injected. Some of these possessed subminimal amounts of thyroid which could be stimulated by the thyrotropic substance present in the growth hormone extract. Those rats which showed no growth response to injections could not be kept alive longer than 45–60 days, the usual result with rats thyro-parathyroidectomized at birth. They were autopsied when it was apparent that they could be kept alive no longer. The gonads were weighed, fixed in Bouin's solution and stained in hematoxylin and eosin. The tracheal region of each experimental animal was serially sectioned and examined for thyroid remnants. Skeletons were preserved in 95% alcohol and later cleared in KOH and glycerine and stained with alizarin (8).

The sex glands and skeletons of the rats described above were subjected to comparison with those of rats whose growth curves have been previously described (5), i.e., untreated newborn thyroidectomized rats and those treated with pituitary implants.

[2] The anterior pituitary growth extract was obtained through the courtesy of Dr. J. A. Morrell of E. R. Squibb & Sons, New Brunswick, N.J.

RESULTS

Fourteen newborn thyro-parathyroidectomized animals in which no thyroid remnant was found did not respond to growth hormone (table 1). They continued on the growth plateau as do uninjected operated rats (5). They showed the same tendency toward intestinal stasis but appeared healthy until the stasis became acute. Growth hormone in no way relieved this condition. Five rats with very small thyroid remnants failed to grow. These began to show signs of sickness and died within a week after the beginning of growth hormone injections.

Of 16 animals thyro-parathyroidectomized at birth which responded to growth hormone injections, thyroid remnants were found in all but one. The remnants in each case were composed of highly stimulated follicles as judged by greatly heightened

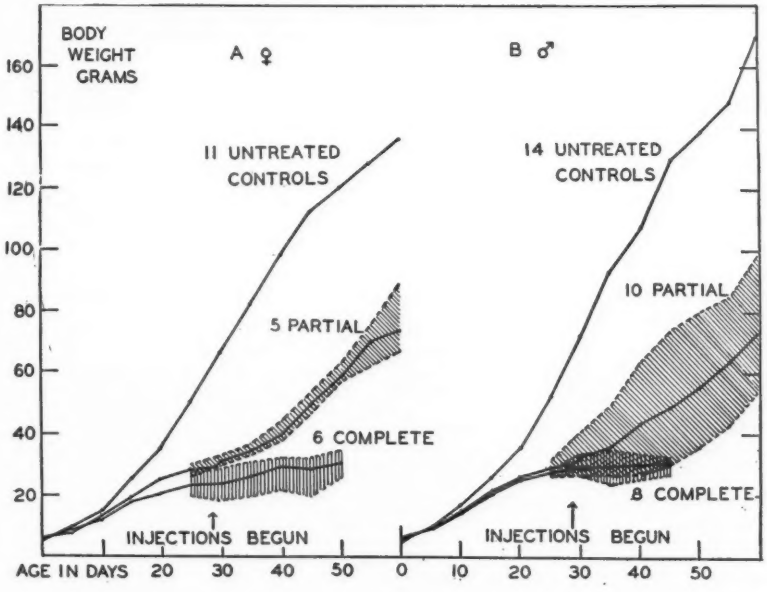

Fig. 1. NEWBORN THYROIDECTOMIZED RATS RESPONDED TO GROWTH HORMONE INJECTIONS ONLY WHEN A THYROID REMNANT WAS PRESENT. Shaded areas indicate the weight ranges of experimental animals.

epithelium and the presence of little or no colloid. The amount of growth response was roughly proportional to the size of the thyroid remnant. In one animal which grew it was not possible to establish microscopically that a thyroid remnant was present though the tracheal region and the thymus were both examined serially. Since thyroid tissue has been known to exist in various abnormal positions, especially after operation, it is probable, judging from the animal's growth, alertness and lack of intestinal stasis that some thyroid tissue was present which was not included in the material sectioned.

Figure 1 shows the growth curve averages for females and males, including the range for experimental animals. The range for females possessing thyroid remnants and therefore responding to growth hormone was very small, but was much greater for the corresponding males. The male group included a few animals with very small remnants at autopsy. Since these remnants were probably even smaller before the thyrotropic factor present in the extract affected them, the delay in growth response

is probably attributable to insufficient thyroid during the first 2 weeks of injection (fig. 1, B). Animals without any thyroid remnants at all failed to show significant departure from the plateau and could not be kept alive as long as those with the smallest remnants.

By about 22 days of actual age, the skeletons of rats thyroidectomized at birth have developed ossification equivalent to that in a normal rat aged 14 to 16 days (9). In the absence of a thyroid remnant ossification does not progress beyond that stage.

TABLE 2. THE EFFECT OF VARIOUS ANTERIOR PITUITARY TREATMENTS ON THE GONADS OF RATS THYROIDECTOMIZED AT BIRTH

Treatment	Males			Females		
	No. of cases	Gonad wt. / Body wt.	Histology	No. of cases	Gonad wt. / Body wt.	Histology
		%			%	
Thyroidectomized control, no AP	5	0.50±0.066	No advance over normal 23-day stage	5	0.019±0.0077	Small follicles only
Completely thyroidectomized, growth ext.	4	0.52±0.16	No advance over normal 23-day stage	3	0.024±0.0005	Small follicles. No corpora. Interfollicular cell hypertrophy
Incompletely thyroidectomized, growth ext.	5	1.78±0.56	Advancing stages of spermatogenesis depending on size of thyroid remnant.	5	0.021±0.0036	Corpora as well as follicles
Completely thyroidectomized, AP implants	3	2.05±0.74	No significant advance in spermatogenesis over 23-day stage. Interstitial hypertrophy.	1	0.111	Numerous corpora
Incompletely thyroidectomized, AP implants	3	2.41±0.37	Advancing stages of spermatogenesis incl. some adult sperm	1	0.091	Numerous corpora
Untreated normal control litter mates	5	0.97±0.087	Normal sperm development	6	0.025±0.0046	Normal

The rats of the present series which did not grow in spite of growth hormone injections did not show any advance in skeletal development or differentiation over untreated operated animals. The ventral ribs remained unossified and the epiphyses of long bones were unossified or only just beginning ossification. The skull proportions remained infantile.

Thyroidectomy at birth caused marked retardation of gonad development. The testes of operated animals (complete removal of thyroid), after 40–60 days, had an average weight relation to the body of 0.50% ±0.066, the ovaries, 0.019% ±0.0077 (table 2). Early testis development proceeded more slowly than the normal, reached the normal 23-day stage at 30 days or over, and thereafter remained at that stage. The ovaries showed follicular development with antra, but no large follicles. Corpora lutea have not been observed in any of the ovaries.

The response of the gonads of completely thyroidectomized rats to growth hormone was not the same as to pituitary implants. Although the growth extract used admittedly contains unassayed amounts of gonadotropic as well as thyrotropic factors, neither the testes nor the ovaries showed an increase in weight (table 2). Histologically the testes remained entirely unstimulated, but the ovaries responded with some interfollicular cell hypertrophy. With anterior pituitary implants, however, the ovaries and the testes increased greatly over those of untreated operated animals in absolute weight and even over the normal in proportion to body weight (table 2). The testes showed no significant advance in spermatogenesis, the size increase being due to increase of interstitial material. Priapism was characteristic of these animals. The ovaries contained numerous large corpora as well as follicles. In short, the spermatogenic elements of the testes responded neither to anterior pituitary implants nor to anterior pituitary extract except in the presence of a thyroid remnant; the ovary responded to implants with lutein development whether thyroid was present or not.

DISCUSSION

The results of the present experiment show that rats completely thyroidectomized at birth are incapable of response to the anterior pituitary growth substances at present available. Since these findings are different from those obtained when rats are thyroidectomized at 35 to 45 days (2), the age at which the animal is deprived of its thyroid appears to be the important factor. The age of the animals may also account for the success in producing acceleration of ossification of the epiphyseal line by pituitary growth substance in the guinea pigs thyroidectomized by Silberberg and Silberberg (3), since guinea pigs of 120 to 180 gm. are likely to be several weeks old, as well as being more advanced in development at birth than the rat. Even normal newborn rats are much less responsive to hypophyseal hormones than older rats (10, 11). The influence of the thyroid at an early age must be, at least in part, responsible for the development of sensitivity of the tissues to growth substance.

In a previous report (5) it was pointed out that rats thyroidectomized at birth were qualitatively as well as quantitatively different from those operated later (7 or 14 days), since a growth plateau is reached and maintained in the former after 3 weeks, while the latter continue to grow though at a rate slower than normal. The skeletal age of rats thyroidectomized at 7 days progresses beyond that ever attained in newborn-thyroidectomized rats (9). The skeleton of rats thyroidectomized at birth lacks the ability to develop beyond a skeletal age of about 15 days. Thus the absence of the thyroid during the first postnatal week is associated with the failure of certain developmental patterns to appear at all. If the newborn rats are completely thyroidectomized, pituitary growth substance does not behave as a substitute for thyroid for reestablishing progress in osseous development.

The gonads of rats thyroidectomized at birth, in contrast to the skeleton, exhibit a partial ability to respond to the pituitary substances used. The spermatogenic elements failed to be stimulated at all, either by implants or by the gonadotropic substance present in the extract. The interstitial portion of the testis responded to implants but not to the growth extract. The ovaries of untreated newborn operated animals showed complete absence of lutein tissue. After implants luteinization occurred. The interfollicular cell hypertrophy found after injections of growth extract could not be interpreted as true luteinization.

The dependence of the infantile rat, a mammal, upon its thyroid contrasts with the relative independence of amphibians until the time of metamorphosis (12, 13). Pituitary removal in both rats and amphibia (14, 15) results in growth retardation. It has therefore been suggested (1, 2) that growth retardation following thyroidectomy in rats is caused primarily by dysfunction of the pituitary due to absence of thyroid

stimulus. However, Smith (16) found that growth need not be considered an effect of the pituitary alone, since thyroid in addition to hypophyseal growth extract resulted in a synergistic action when administered to thyroidectomized, hypophysectomized rats (operation 40–50 days), in which there could be no question of stimulation of the animal's own pituitary by the thyroid given. In the newborn rat, however, the thyroid appears to play a more than synergistic role since the capacity to respond to certain hypophyseal substances remains undeveloped without it. Apparently it is not possible to generalize concerning the relation between the pituitary and the thyroid and their respective potencies without considering the ages of the individuals in which such relationship is described.

SUMMARY

Of 128 rats, thyro-parathyroidectomized at birth, 35 were injected after weaning time with 3 U daily of anterior pituitary growth extract. The growth curves, skeletons and gonads of these animals were compared with those of untreated newborn thyroparathyroidectomized rats and those treated with anterior pituitary implants.

Growth was not induced by the extract or by pituitary implants in animals which were completely without thyroid.

The skeleton of rats thyroidectomized at birth progresses to and remains at a skeletal age of about 15 days whether untreated, given growth extract or pituitary implants.

The testes of untreated newborn thyroidectomized animals do not progress beyond an equivalent of the normal 23-day stage. Spermatogenic elements responded neither to anterior pituitary implants nor to growth extract if no thyroid was present. Increase in interstitial material followed pituitary implants.

The ovaries of untreated or growth extract treated newborn thyroidectomized rats were consistently without corpora lutea. The ovaries responded with luteinization to pituitary implants whether thyroid was present or not.

It is suggested that in the early postnatal life of the rat the thyroid is responsible for the development of the capacity of the organism to respond to other hormones.

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EFFECT OF ESTROGENS, GONADOTROPINS AND GROWTH HORMONE ON MAMMARY GLANDS OF HYPOPHYSECTOMIZED RATS[1,2]

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ALTHOUGH much experimental evidence has been presented to show the growth-stimulating effects of ovarian hormones on the mammary gland of normal and gonadectomized rats, conflicting results have been reported when hypophysectomized rats have been employed.

Ruinen (1), de Jongh (2), and Freud and de Jongh (3) stated that good mammary growth could be obtained in hypophysectomized rats treated with ovarian hormone. Nelson (4) reported that mammary proliferation in the hypophysectomized rat in response to estrogen was erratic but in general much less marked than in spayed animals.

Reece, Turner and Hill (5) first showed that growth of the mammary gland in hypophysectomized rats could not be obtained as a result of estrogen administration even though the ovaries were present. Similar results have been reported (6–9). In addition, it has been noted (10) that even well nourished hypophysectomized rats did not show mammary development following the injection of large doses of estrogen for a 28-day period.

Herold and Effkemann (11) found estrogen administration ineffective on the mammary glands in male and spayed rats in which the pituitary stalk had been sectioned. More recently, however, reproductive cycles, ovulation, mating, pregnancy and lactation were reported normal after section of the pituitary stalk in the rat (12).

The injection of a combination of anterior pituitary gland extracts with an estrogen in an attempt to elicit mammary growth has met with varying degrees of success. Seyle and Collip (13) reported that crude hypophyseal extracts capable of maintaining other endocrine glands did not aid estrogen in stimulating mammary growth in the rat. On the other hand, Nelson and Tobin (6) observed that hypophysectomized rats treated with a crude pituitary extract and an estrogen showed well developed mammary glands. Mammary growth in hypophysectomized rats injected simultaneously with the growth complex and estrogen was found dependent on the increase in body weight (9).

This study was undertaken in order to re-examine the effects of exogenous estrogens alone and in conjunction with growth hormone preparations and endogenous estrogens (produced by gonadotropins) on mammary growth in hypophysectomized rats.

METHODS

Hypophysectomy of albino or piebald rats was carried out by the parapharyngeal route, and completeness of the operation was checked by sectioning the sella turcica.

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[1] Read at the twenty-fifth annual meeting of the Association for the Study of Internal Secretions on May 2, 1941 in Atlantic City, N.J.

[2] Journal Series Paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Husbandry.

Weight changes were noted of the body, thyroid, adrenals and gonads, and the endocrine organs were further examined microscopically. A mammary gland from a similar location was removed from each rat and stained in toto.

RESULTS

Exogenous estrogens and mammary growth. Six 33-day-old female rats were spayed; 5 days later they were hypophysectomized, and after another period of 5 days, 3 were injected with 20 R.U. of estradiol benzoate[3] daily for 10 days. The animals were found to be completely hypophysectomized as indicated by body weights, endocrine weights, and the absence of pituitary fragments in the sellae turcicae. In no instance were the mammary glands stimulated. The glands consisted of only a naked duct system in both groups.

This experiment was then repeated on 7 castrated rats of the same age except that 1 rat did not have the pituitary removed. Three of the operated rats and the intact animal received the same estrogen treatment as above, the 3 remaining rats served as controls. The results showed the mammary glands of the 3 operated controls to consist of only naked ducts (fig. 1). One of the 3 injected rats had about ⅛ to ½ of the hypophysis remaining and the mammary gland appeared to be proliferating rapidly (fig. 2). The degree of proliferation, however, was not as great as that of the normal rat injected with estrogen (fig. 3). The 2 remaining hypophysectomized injected animals possessed mammary glands indistinguishable from the operated controls (fig. 4).

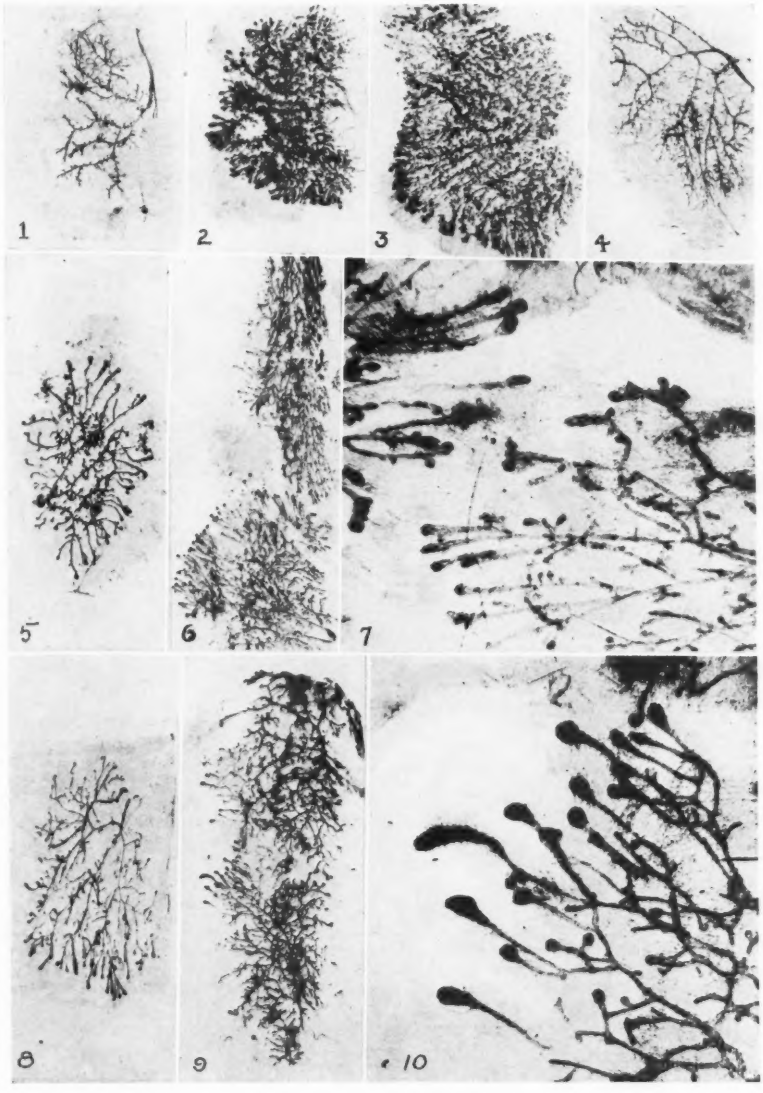
Similar studies were then made on eight 26-to 32-day-old male rats. The animals were hypophysectomized and beginning on the following day 5 of the 8 rats received 50 R.U. of estrogen[4] daily for 11 days. The remaining 3 animals received no treatment. At autopsy, the operation was found to be complete in only 2 of the 3 controls. In these 2, short naked ducts comprised the mammary parenchyma. The mammary gland of the incompletely operated control contained a few enlarged end buds and numerous but small lateral buds.

Only 1 of the 5 experimental animals had all the pituitary gland removed, and the mammary parenchyma of this animal consisted of a restricted naked duct system. What appeared to be nests of anterior pituitary cells were observed in the other rats in the sella turcica where the infundibulum pierced the meninges. One mammary gland showed only a single enlarged end bud with the lateral buds still present. In another gland from this group (fig. 5) the duct system was considerably extended, containing many enlarged end buds and normal appearing lateral buds.

Eight 42-day-old male rats were hypophysectomized and injections delayed for 221 days. The rats then received 50 R.U. of estradiol benzoate[5] subcutaneously daily for 16 days. Five of the animals were found to have been completely hypophysectomized, and their mammary glands consisted of restricted naked duct systems. The operation was incomplete in the remaining 3 animals, and their mammary glands showed typical estrogen stimulation. These results are summarized in table 1.

Endogenous estrogens from gonadotropins on mammary growth. The pituitary gland was removed from 22 30-day-old female rats and after 6 days 20 of these rats received injections of gonadotropins for 12 days. The hormones used were a pregnancy urine preparation,[6] a urinary gonadotropic hormone from menopause urine containing both the follicle stimulating and luteinizing hormones,[7] and a follicle

[3] Progynon-B, Schering.
[4] Amniotin, Squibb.
[5] Progynon-B, Schering.
[6] Follutein, Squibb.
[7] Gamone, Squibb.



MAMMARY GLANDS OF MALE RATS. Figures illustrating the entire gland ×2, all other figures ×13. Fig. 1. Castrated hypophysectomized. Fig. 2. Castrated partially hypophysectomized (⅓–½ gland) injected with estrogen. Fig. 3. Castrated injected with estrogen. Fig. 4. Castrated hypophysectomized injected with estrogen. Fig. 5. Castrated partially hypophysectomized (minute fragment) injected with estrogen. Fig. 6 and 7. Hypophysectomized injected with growth hormone plus estrogen (immediate treatment). Fig. 8, 9, and 10. Hypophysectomized injected with growth hormone plus estrogen (postponed treatment).

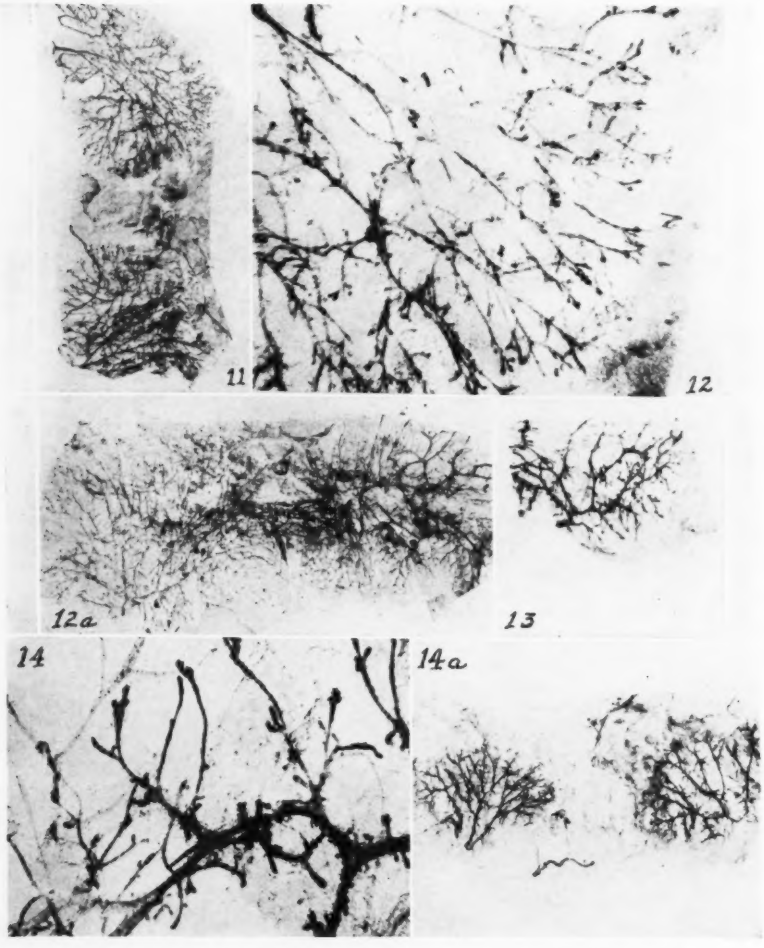


Fig. 11 and 12. Hypophysectomized injected with growth hormone (immediate treatment). Fig. 12a. Hypophysectomized control for immediate treatment. Fig. 13 and 14. Hypophysectomized injected with growth hormone (postponed treatment). Fig. 14a. Hypophysectomized control for postponed treatment.

stimulating hormone prepared from gland sources and containing small amounts of the luteinizing principle.

It was noted that gonadotropic hormones all elicited considerable increase in ovarian weight, while the uterine weights indicated that ovarian hormone production was stimulated in the ovaries of the experimental rats. The lowered adrenal and thyroid weights all indicated the effects of hypophysectomy. The mammary glands from the 4 groups of rats were similar, no proliferation having occurred in a single gland. No evidence of unmistakable hypophyseal tissue was found. The results are summarized in table 2.

Growth hormone and estrogen. Young male rats, ranging in body weight from 100 to 150 gm. were hypophysectomized and injected with growth hormone or growth

TABLE 1. THE INFLUENCE OF EXOGENOUS ESTROGEN ON MAMMARY GLANDS OF HYPOPHYSECTOMIZED RATS

Treatment	No. rats	Sex	Average gain or loss in body wt.	Thyroid wt.	Adrenal wt.	Mammary gland growth
			gm.	mg.	mg.	
Castrated-hypophysectomized	3	F	+12	4.6	10.7	None
Castrated-hypophysectomized—20 R.U. estradiol benzoate[1] daily 10 days	3	F	−20	5.3	11.9	None
Castrated-hypophysectomized	3	M	− 8	5.4	8.4	None
Castrated-hypophysectomized—20 R.U. estradiol benzoate[1] daily 10 days	2	M	−16	5.1	8.3	None
Castrated-incomplete hypophysectomy	1	M	+ 7	7.5	28.1	Present
Castrated	1	M	+21	7.6	38.5	Present
Hypophysectomized	2	M	+ 9			None
Incomplete hypophysectomy	1	M	+24			Present
Hypophysectomized—50 R.U. estrogen[2] daily 11 days	1	M	0			None
Incomplete hypophysectomy — 50 R.U. estrogen[2] daily 11 days	4	M	+5			Present
Incomplete hypophysectomy — 50 R.U. estradiol benzoate[1] daily 16 days	3	M	+49	9.2	11.5	Present
Hypophysectomized — 50 R.U. estradiol benzoate[1]	5	M	+ 3	4.1	5.0	None

[1] Progynon-B.
[2] Amniotin.

hormone in combination with estradiol benzoate for 12 to 15 days. Daily doses of 50 R.U. of estradiol benzoate, subcutaneously, and 0.5 cc. of growth hormone, intraperitoneally were injected. Uninjected hypophysectomized rats were prepared as controls. In some experiments, several weeks elapsed before injections were begun (postponed treatment), in others, injections were begun within 12 hours after the operation.

The mammary glands of the rats receiving growth hormone plus estrogen indicated renewed growth of the end buds and enlargement of the ducts. The effect is fairly characteristic in that the ends of the ducts in the peripheral region of the gland appear club-shaped (fig. 6–10). When growth hormone alone is injected, the ducts and lateral and end buds do not regress to the degree that is characteristic of the uninjected hypophysectomized controls (fig. 11, 12, 12a). In the 'postponed' treated rats receiving growth hormone alone, new growth was obtained in the lateral buds

TABLE 2. THE INFLUENCE OF ENDOGENOUS ESTROGENS ON MAMMARY GLANDS OF HYPOPHYSECTOMIZED RATS

Treatment	No. rats	Average gain or loss in body wt.	Ovarian wt.	Uterine wt.	Adrenal wt.	Mammary gland growth
		gm.	mg.	mg.	mg.	
Hypophysectomized	3	+6	4.5	20	8.9	None
Hypophysectomized—385 R.U. PU[1]	5	+5	16.8	96	8.0	None
Hypophysectomized—6 mg. urinary gonadotropin[2]	6	+4	35.9	78	8.0	None
Hypophysectomized—0.5 mg. FSH	8	0	57.5	165	8.5	None

[1] Follutein.
[2] Gamone.

and to a lesser extent in the end buds, but in almost all cases the main ducts were definitely thickened (fig. 13, 14, 14a).

Variation in the degree of response of the mammary glands to these injections was quite evident. Of the group which received growth hormone plus estrogen, only 1 rat failed to respond by the characteristic growth of the terminal buds. Incidentally, this rat lost the greatest amount of weight during the course of injections (38 gm.).

TABLE 3. GROWTH HORMONE ALONE OR IN COMBINATION WITH ESTROGEN INFLUENCING BODY WEIGHT AND MAMMARY GROWTH IN HYPOPHYSECTOMIZED MALE RATS (IMMEDIATE TREATMENT)

Exp. no.	Treatment	No. rats	Average gain or loss in body wt.	Thyroid wt.	Adrenal wt.	Mammary glands
			gm.	*mg.*	*mg.*	
	Hypophysectomized control	5	−16	9.0	12.3	Naked duct system
106	Growth hormone plus estrogen	4	−24	—	14.8	Characteristic Estr. stimulation. 1 except.
	Growth hormone	3	− 5	—	11.9	Incomplete atrophy possibly new duct buds
107	Growth hormone plus estrogen	4	− 9	8.8	11.6	Estrogen stimulation
	Growth hormone	3	+14	8.0	11.0	Incomplete atrophy
108	Growth hormone plus estrogen	5	− 5	8.7	9.7	Estrogen stimulation
	Growth hormone	5	+16	8.1	7.9	Incomplete atrophy
	(Postponed treatment)					
89	Growth hormone plus estrogen	3	+ 7	6.9	11.7	Estrogen stimulation
	Growth hormone	2	+28	7.6	12.0	New growth
83	Growth hormone plus estrogen	7	+ 4	6.6	7.5	Estrogen stimulation
	Growth hormone	7	+24	5.9	6.6	New growth
	Hypophysectomized control	3	− 5	5.8	7.6	Naked duct system

In general it could be said that those rats which gained the most or lost the least in body weight had the greatest proliferation of their mammary glands. It is to be noted that the loss of body weight, in all cases but one, did not necessarily mean a failure of estrogen to stimulate the mammary glands.

From the results in table 3, it is seen that estrogen can antagonize the effect of growth hormone in stimulating body growth. Sometimes this was revealed by a still greater loss of weight in those rats in which the hormone was insufficient to check the normal loss which follows hypophysectomy. More often, it prevented the normal increase in body weight which ordinarily follows growth hormone injections.

Microscopic examination of the thyroids indicated that thyrotropic hormone was present in the growth hormone. In all rats receiving growth hormone, the epithelium showed hyperplasia. Columnar cells lined the follicles and numerous colloid absorption vacuoles were sometimes present. No distinction could be made between the thyroids of those rats which received estrogen in addition to the growth hormone and

those which did not. There was no apparent change in the weight of the thyroids as a result of the hormone treatment.

The adrenal glands neither increased in weight with the postponed injections nor did the growth hormone maintain adrenal weights when injections were begun immediately. Microscopically, no outstanding improvement of the cortex was observed in the rats receiving growth hormone, although the general impression was that the cortical cells in some cases were more like those found in normal rats. This was particularly true in the zona glomerulosa and zona fasciculata where many cells remained normal in appearance. The estrogen injections had no effect on the adrenal cortex in this experiment.

Gonadotropic hormone was present in the growth extract as revealed by partial maintenance of the testis and accessories. It was noted that estrogen definitely antagonized this contaminating gonadotropic hormone in the operated rats. The details of these findings will be considered at a later time.

A study of the sectioned sellae turcicae revealed that in several cases, fragments of hypophseal tissue were present. These rats were not included in the data. Gross examination of the sellae turcicae is not sufficient to determine completeness of hypophysectomy because fragments of the gland which are too small to be observed with the binocular magnifier are sufficient to give mammary growth with estrogens. Sometimes isolated nests of cells which were sufficient to allow estrogens to exert their effect seemed, from their position, to defy removal by the ordinary technic of hypophysectomy.

DISCUSSION

The evidence presented here confirms previous conclusions that the hypophysis is necessary for estrogens to induce growth of the mammary glands. Estrogen will neither maintain the glands in recently hypophysectomized rats nor will it induce new growth in glands that have undergone atrophy.

The possibility that endogenous estrogen could induce mammary growth in hypophysectomized rats has proved negative, PU, hypophyseal gonadotropic hormone rich in the follicle stimulating fraction, and menopause urine gonadotropin[7] all failed to influence the mammary glands even though adequate amounts of estrogens were made available.

The growth hormone, however, seems to supply the necessary substance which enables the mammary glands to respond to estrogen. Nathanson, Shaw and Franseen (9), using a different growth hormone preparation, found essentially the same result. These workers state that unless growth hormone at least maintains or causes marked growth in the rat, the estrogens do not exert a marked effect on the mammary glands. In our experiment, our rigorous criterion has been the presence or absence of such growth that is characteristic of the effect of estrogen in male rats (compare fig. 5 and 8). Growth hormone alone can effect the mammary glands, but estrogen stimulation, so noticeable in male rats, is distinctly different. In general it was noted that the more effective the growth hormone, the more responsive were the mammary glands to estrogen. The same principle holds true for varying degrees of partial hypophysectomy.

The question arises as to what fraction in the growth hormone may be responsible for this reaction of the mammary glands to estrogen. Gonadotropic hormone was present but its effect can be ruled out by the experiments summarized in table 2. Thyrotropic hormone was definitely present and no doubt some adreno-corticotropic hormone, although probably in very small amounts. Whether or not either one of these hormone fractions or both could still react in the same way with estrogen remains to be determined. Likewise further experiments using cortin and thyroxin should be

tried, although some previous work has shown that these substances are not effective (7).

The concept that estrogen causes growth of the mammary gland by inducing the hypophysis to form and release a hypophyseal factor (14) can be viewed in another light. In the castrated hypophysectomized female rat, it was recently demonstrated (15) that hypophyseal tissue can induce growth of the mammary gland but that estrogen did not increase this mammogenic factor. In view of the fact that growth hormone alone induced slight mammary growth one might state that either estrogen facilitated the mammogenic effect of the growth hormone or that the growth hormone facilitated and was responsible for the effect of estrogen.

According to the concept of Astwood, Geschickter and Rausch (8) estrogen stimulation of the rat mammary gland depends on the state of 'nutrition' of the animal. The application of this reasoning to the failure of estrogens to induce mammary growth in hypophysectomized rats leaves some points unexplained. The young hypophysectomized rats in our experiments receiving gonadotropic hormone grew slightly, yet their mammary glands were not influenced by the endogenous estrogens. Estrogen however, stimulated mammary growth in the presence of growth hormone, in spite of weight losses. The 'nutritional state' may play some part in the degree of response, but the concept of the nutritional state must first be clarified by the exponents of this theory. Estrogens are antagonistic to the effects of growth hormone on growth as revealed by our data and also to the growth hormone content of the hypophysis as previously shown (15). Estrogens were found to antagonize the injected gonadotropic hormone (such as was found in the growth hormone complex) but seemed to have no effect on the thyrotropic or adreno-corticotropic factors.

In regard to partial hypophysectomy, some of the results were of pertinent interest. In 2 cases where at least one-third of the gland remained, the effects of hypophyseal deficiencies as revealed by continued body growth, thyroid, adrenal and mammary gland structure were not as great as in one rat with approximately one-tenth of the gland remaining. The degree of vascularity of that one-tenth was estimated to be greater than that in the other 2 cases where a larger amount of hypophyseal tissue remained. Often when the sella turcica was examined, a large group of epithelial cells closely applied to the pituitary stalk was found and at first glance was thought to be hypophyseal tissue. In most of these cases all other data pointed to complete hypophysectomy, and it was suspected that these were cells of the pars tuberalis.

SUMMARY

Estrogen administration failed to induce mammary stimulation in castrated hypophysectomized, hypophysectomized male and spayed hypophysectomized rats.

Endogenous estrogens, produced by the injection of gonadotropic hormones in hypophysectomized female rats, exerted no influence on the mammary gland even though the body weights of these animals increased or remained constant during the course of treatment.

Growth hormone injected into hypophysectomized male rats produced slight mammary growth, but the simultaneous administration of estrogen stimulated the growth of the duct system. It is suggested that either estrogen facilitates the action of the mammogenic factor contained in the growth hormone or that the growth hormone with its mammogenic factor facilitates and permits the action of estrogen on the mammary glands.

Estrogen inhibited the normal effect of growth hormone in inducing an increased body weight in the hypophysectomized male rat. Estrogen, however, did not influence

the effect of the thyrotropic or adreno-corticotropic hormones which were present in the growth hormone complex.

Hormones used in these experiments were supplied as follows: Follutein, Gamone, Amniotin and the growth hormone preparation by E. R. Squibb & Sons, through the courtesy of Doctors J. A. Morrell and J. S. De Frates, Progynon-B by the Schering Corporation through the courtesy of Doctor Erwin Schwenk.

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EFFECT OF THYROIDECTOMY OF YOUNG MALE GOATS UPON CERTAIN AP HORMONES[1,2]

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THYROIDECTOMY of very young animals causes a gradual cessation of growth (1–5). The injection of thyroxine or the feeding of thyroid has caused a restoration of the growth rate, (2, 4, 5, 6). As the secretion of the thyroid gland influences general body metabolism, it might be suggested that the reduction in the growth rate is an expression of reduced cellular mitotic activity of the somatic cells. This would account for a reduced rate of growth, but not for the early complete cessation of body growth. It is also possible that the lack of thyroid secretion might affect the rate of secretion of some or all of the pituitary hormones including those factors influencing somatic growth.

As the rate of growth is of great economic importance in domestic animals, anything which will contribute to our understanding of the endocrine control of growth is of interest. For this reason it seemed desirable to determine the effect of thyroidectomy upon the concentration in the pituitary of various AP hormones in experimental animals. The present report concerns the effect of thyroidectomy of young male goats upon the concentration of the lactogenic, thyrotropic, gonadotropic and the blood sugar raising factors. For purposes of comparison groups of normal animals of comparable age, but larger because of continued normal growth, and of younger animals of comparable body weight were selected. Additional groups of animals are now being prepared for the study of other pituitary factors.

Houssay, Novelli and Sammartino (7), Holhweg and Junkmann (8), and Kuschinsky (9) studied the effect of thyroidectomy of rats, guinea pigs and dogs upon the thyrotropin content of their pituitaries as measured by the change of the thyroid of immature guinea pigs. They were unable to distinguish a definite trend in the limited number of animals studied and by the assay methods employed. Of the more recent studies, it is interesting to note that Lebedewa (10) reported a decreased potency of thyrotropin in thyroidectomized rats in comparison to normals. Zeckwer (11) confirmed these observations when comparison was made with controls of the same age. As the controls were larger and their pituitaries larger, the comparison with the cretinous pituitaries might more properly be compared with controls of the same weight. Turner and Cupps (12) noted a reduction in thyrotropin in the male rat pituitary whereas in the female the hormone content was maintained if not slightly increased. As these workers (13) reported that the female rat pituitary contained only 62% as much thyrotropin as the male per gram of fresh tissue, the reduction in the males and not in the females may be explained. In the rabbit, Chen and Van Dyke (14, 15) noted significantly more thyrotropin in the female AP 3 months or longer after thyroidectomy, whereas in male rabbits the total amount of hormone available was not affected.

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While there is considerable indirect evidence that thyroidectomy in some animals reduces the amount of gonadotropic hormones because it interferes with the reproductive cycle, there is surprisingly little direct work upon the pituitary. Evans and Simpson (16) thyroidectomized young female rats and after 5 weeks assayed their pituitaries in comparison with litter-mate controls. The cretinous pituitaries were reported to be less effective than the normals in causing sexual maturity in immature rats. Smith and Engle (17) at the same meeting reported on the effect of long continued thyroidectomy (200 days or more) of female rats. In comparison with normal litter-mate controls, no difference was noted in the gonads or uterus when the two types of pituitaries were implanted into immature mice.

Van Dyke and Chen (18) found the concentration of the ovulation producing factor for rabbits reduced in thyroidectomized rabbit pituitary in comparison with litter-mate controls. P'an (19) reported that the gonadotropic content of the pituitary of normal and castrate rats and of rabbits was decreased after thyroidectomy.

Only one preliminary report on the lactogenic hormone is available. McQueen-Williams (20) reported that thyroparathyroidectomized male rat pituitaries contained less lactogen than normal.

EXPERIMENTAL

Procedure. A group of 8 male kids were thyroidectomized between the age of 5 and 24 days. They were then killed when over 4 months of age. At that time the body weight was obtained and the following glands and organs weighed: liver, pituitary body, anterior pituitary, testis, adrenal and pancreas. The liver fat was extracted and weighed and the blood fat was determined in oxalated blood plasma by the Allen method (21). Blood sugar was determined in a zinc hydroxide blood filtrate by the Shaffer and Somogyi method (22).

For comparison a group of normal kids was killed when at approximately the same body weight as the thyroidectomized animals, and a second group at about the same age (table 1).

As soon as the pituitaries were weighed they were frozen until ready for assay. In preparation for assay, the anterior lobes of each group were macerated in a small agate mortar until fine enough to pass through a 22-gauge needle and then suspended in distilled water so that each cc. contained 100 mg. of the fresh pituitary. From the stock solution aliquot portions were taken and further diluted as required for the various assays.

The lactogenic hormone was assayed, using common pigeons, by the intradermal (micro test) method. A unit has been defined as that amount of hormone which when injected intradermally over the crop gland of 20 common pigeons will elicit a minimum response in $50 \pm 11\%$ of the pigeons (23). The micro-units of lactogenic hormone are converted to international units (24). The thyrotropic and gonadotropic hormones were assayed with day-old male White Leghorn chicks (25, 26). The blood sugar elevating factor of the AP was determined by a method described by Bergman and Turner (27) which calls for an increase of 50% in glucose in the blood of immature male guinea pigs (180–220 gm.).

Observations. When male goats are thyroidectomized soon after birth, the rate of growth soon slows down and finally stops (6) It will be noted in this study that normal male kids between 2 and 3 months old weighed an average of 22.2 lb. in contrast to 18.1 lb. for the thyroidectomized animals between 4 and 5 months old. The animals of comparable age weighed an average of 32.0 lb. As even the young normal animals are 22.5% heavier than the experimental animals, the body weight and various gland weights of the thyroidectomized group were increased by the factor 1.225 for purposes of comparison. This may introduce a slight error in data for organs which do not increase in direct proportion to body weight. On this basis it is seen that the

TABLE 1. COMPARISON OF ORGAN WEIGHT OF NORMAL AND THYROIDECTOMIZED MALE (KID) GOATS

Animal No.	Body weight	Pituitary body	Anterior pituitary	Testis	Adrenal	Pancreas	Thyroid	Liver	Liver Fat		Blood sugar	Plasma fat	Age	Remarks
									%	Total gm.				
	lb.	mg.	mg.	gm.	mg.	gm.	mg.	gm.			mg. %	mg. %	mo.-d.	
71	17.0	137.4	120.3	7.9	702.7	10.9	546.2	214.3	6.95	14.89			2-17	Small normals
92	17.5	166.3	137.3	4.9	785.0	13.0	802.6	229.5	6.79	15.58				
12	22.5	174.1	145.2	46.9	807.0	22.6	394.7	215.9	7.53	16.25	48.0	125.2	3-26	
52	22.9	150.8	122.7	14.2	762.7	19.9	839.3	223.9	7.12	15.94	48.0	170.0	2-5	
90	23.0	202.0	182.2	20.4	922.1	13.6	580.8	331.5	7.03	23.30			2-23	
56	23.1	177.4	146.5	16.2	891.8	11.4	795.7	300.3	9.56	28.70	52.0	137.8	2-4	
62	23.2	158.4	133.1	9.9	882.0	16.3	784.1	281.2	6.90	19.40	55.0	164.1	2-0	
93	24.5	151.4	133.8	12.9	785.4	15.7	752.7	247.7	7.04	17.44				
42	26.9	202.8	182.3	22.9	1011.6	19.0	1292.7	305.5	10.36	31.59	56.0	280.2	2-16	
mean	22.2	169.6	144.8	17.4	838.9	15.8	754.3	261.1	7.70	20.34	51.8	175.4		
91	30.0	290.0	249.2	33.0	795.0	19.5	1131.8	356.8	7.30	26.04	75.5	337.2	4-11	Larger normals
64	30.5	219.4	187.8	26.2	754.7	16.0	1105.5	347.9	7.37	25.64	83.8	231.8	4-14	
79	35.5	315.8	276.4	23.9	897.2	16.9	8263.0	403.4	6.96	28.07	67.3	230.4	4-14	
mean	32.0	275.0	237.8	27.7	815.6	17.4	3500.1	369.3	7.21	26.58	75.5	266.4		
8	16.5	111.8	94.3	3.6	851.6	17.1		143.9	8.30	11.94	36.0	141.0	4-0	Thyroidectomized
6	17.0	174.3	142.6	5.0	1005.6	7.7		211.8	7.87	16.67	44.0	114.2	4-8	
51	7.5	168.7	139.7	6.2	820.7	17.6		155.9	6.89	10.74	31.3	158.5	4-20	
7	18.0	145.6	116.6	12.12	684.2	15.8		216.3	7.35	15.90	87.0	115.0	4-0	
19	18.0	205.5	171.4	23.1	613.3	13.6		179.5	8.52	15.29	43.0	72.2	3-0	
54	18.5	260.1	218.2	6.7	678.4	45.9		133.3	5.73	7.64	85.5	138.2	4-21	
5	19.0	150.3	113.2	3.6	1421.3	17.6		146.1	6.98	10.20			4-8	
46	20.5	158.2	143.8	14.2	1010.5	16.8		235.0	8.47	19.90	58.0	199.7	4-9	
mean	18.1	171.8	142.8	10.6	885.7	15.2		177.7	7.51	13.50	48.1	117.3		
Equivalent wt.[1]	22.2	210.4	174.9	12.9	1088.4	18.6		217.1						
% difference		+19.68	+12.2	−34.2	+13.2			−20.3						

[1] As the thyroidectomized kids are lighter than the small normals in body weight, they have been increased by the factor 1.225 for comparison.

pituitary and the adrenal of the thyroidectomized are larger than the small normal controls while the testes and liver are definitely smaller.

The pancreas requires special mention; those of the normal kids were discrete well-formed glands, of a healthy pink color and of firm consistency. The pancreas of thyroidectomized animals had a gross appearance differing markedly from the normal. Instead of a clearly defined gland, it consisted of a soft flabby mass of fatty tissue. Histological examination revealed evidence of marked fatty infiltration and degeneration of acinar tissue, which appeared shrunken and inactive.

The blood sugar level of the experimental animals was similar to that of the small normals whereas the plasma fat was somewhat low.

The assay of the groups of pituitaries revealed that there was an appreciable amount of the lactogenic hormone in the AP of young male goats which increased both in pigeon units per pituitary and in concentration per gram with increasing age and body weight. The AP of the thyroidectomized animals contained more lactogen per pituitary than the small normals and less than the large normals. The lactogen concentration per gram of pituitary of the large normals and thyroidectomized animals was the same (table 2).

Thyrotropic hormone increased with increasing body weight of the normal groups both on the basis of amount per pituitary and the concentration per gram. Here again the units per gram of pituitary in the large normals and thyroidectomized animals were about the same but since the cretinous pituitaries were smaller, the total amount per pituitary was smaller. Thus in the case of the lactogenic and thyrotropic hormones, the concentration of hormone follows chronological age rather than weight.

The assay of the gonadotropic hormones is of special interest because of the low testes weight of the thyroidectomized animals. The chick units of gonadotropin per pituitary increased with increasing body size in the normals whereas the concentration per gram of pituitary was about equal. However, the AP of the thyroidectomized animals was below either group both in amount per pituitary or in concentration per gram. It would appear that the small testes weight is definitely associated with low gonadotropic content of the pituitary and presumably low secretion rate.

The sugar level of the blood is controlled in part by the hormones of the pituitary. The assay employed measures the factor which elevates the blood sugar. It was observed that both the concentration per pituitary and the concentration per gram of pituitary increased with age or weight in the normal animals. The pituitaries of the thyroidectomized animals contained very little of this factor.

DISCUSSION

In a study of the effect of thyroidectomy of mammals a number of variables are present which may influence the results obtained. Of these the age at the time of ablation and the interval between ablation and killing are of greatest importance. Since the present study was concerned with the relation of thyroidectomy to the growth process, the animals were thyroidectomized soon after birth. Second, the time chosen for killing was about 2 months after growth had ceased. In this way it was hoped to measure, if possible, changes in the pituitary hormones caused by the absence of thyroxine which were responsible for growth stasis.

These data contribute further to the theory that the lactogenic hormone is not concerned with the general somatic growth process. The concentration of lactogen in the pituitary is as high as in the animals of comparable age. It does not seem reasonable to suppose it could be a limiting factor in growth. This is interesting in the light of the claims of growth stimulating properties of prolactin by Riddle et al. (28). Evidence of a different character has been presented earlier in opposition to this idea where it was

TABLE 2. EFFECT OF THYROIDECTOMY OF GOATS ON CERTAIN AP HORMONES

No. of animals	Condition of animals	Body wt., av.	AP wt., av.	Lactogen assay				Thyrotropin assay			Gonadotropin assay			Carbohydrate metabolism assay		
				No. pigeons used	Pigeon u[1]/AP.	Pigeon u[1]/gm. AP.	I.U./gm. AP.	No. chicks used	Chick u/AP.	Chick u/gm. AP.	No. chicks used	C.U./AP.	C.U./gm. AP.	No. guinea pigs used	G.P. u/AP.	G.P. u/gm. AP.
		lb.	*mg.*													
9	Small normals	22.2	144.8	20	1448.0	10,000	62.50	18	7.561	52.22	18	7.798	53.85	5	1.261	8.71
3	Large normals	32.0	237.8	20	2972.5	12,500	78.13	10	19.093	80.06	10	12.140	51.05	2	2.778	11.68
8	Thyroidectomized	18.1	142.8	30	1785.0	12,500	78.13	18	11.290	79.06	18	4.203	29.43	5	0.436	3.05

[1] Micro units. One international unit (I.U.)= 160 micro units.

shown that in both the rat (29) and rabbit (30) the level of lactogenic hormone in the male remains low throughout life. On the other hand, in the female, lactogen increases after the period of rapid growth has passed.

The observation of the maintenance of a concentration of thyrotropic hormone in thyroidectomized males comparable with the normal even though the body and AP size was less, conforms fairly well with previous observations. It is difficult to conceive of thyrotropic hormone influencing growth other than through the thyroid (28). Thus the two pituitary hormones which Riddle claims to synergistically influence somatic growth are shown to be present in normal concentrations in the cretinous AP.

In most species, thyroidectomy has been shown to seriously interfere with reproduction. Successful reproduction, however, has been reported in cretinous sheep and goats (1, 3, 6). In sheep thyroidectomy of rams has been reported to affect sperm production (31) whereas in bulls normal semen and sperm were observed (32). In isolated cases of reproductive success, the question of completeness of operation may be raised. The present data indicate low gonadotropic activity of the pituitary and lack of stimulation of the testes.

The available pituitary tissue from thyroidectomized goats permitted an assay of only a single metabolic factor that of the sugar elevating factor which might be concerned in the somatic growth process. One of the writers (33) has suggested the theory that "while there may be a factor which stimulates division of somatic cells, rapid growth is believed to be dependent upon the pituitary factors regulating the metabolism of sugar, fat, protein and mineral matter, for only in their presence can body tissue multiply at a rapid rate." The present data clearly indicate that one AP factor, namely that regulating sugar metabolism is greatly reduced by thyroidectomy. There is also histologic evidence that the pancreas is abnormal and insulin secretion may be affected. The low liver weight is significant also in connection with carbohydrate metabolism as it usually serves as a glycogen depot. Unfortunately liver glycogen was not determined. Liver fat, however, seemed to be present in normal concentration.

From the data presented, it seems evident that thyroidectomy is followed by serious derangement of the glands and organs responsible for carbohydrate metabolism. It is suggested that the inability of the thyroidectomized animal to mobilize sufficient carbohydrate coupled with the low metabolic rate of the cells play an important role in the growth stasis observed.

SUMMARY

A group of 8 male kids was thyroidectomized between the ages of 5 and 24 days, then killed at about 4 months of age after growth stasis had appeared. Their pituitaries were then assayed for the lactogenic, thyrotropic, gonadotropic and sugar elevating factor in comparison with groups of normals of the same weight (but younger) and of the same age (but of normal weight).

Both the lactogenic and thyrotropic hormones were present in the cretinous pituitaries in concentrations comparable to the normals of similar age; thus they do not appear to be the cause of the growth stasis. This observation is believed to be significant in the light of Riddle's claim that these two factors have a synergistic influence upon somatic growth.

The gonadotropic hormone was found to be present in low concentration and the testes showed lack of stimulation, averaging lighter than the youngest group of normal controls.

Of the hormones of the pituitary which are concerned with metabolism, only the

sugar elevating factor was studied. It was shown to be extremely low in concentration in the cretinous pituitaries. However, the histological abnormality of the pancreas and the low liver weight indicated that thyroidectomy is followed by serious derangement of the glands and organs concerned with carbohydrate metabolism and suggested the possibility that this condition coupled with the low metabolic rate of the somatic cells plays an important role in the growth stasis observed.

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THYROTROPIC HORMONE CONTENT OF RABBIT PITUITARY DURING GROWTH[1,2]

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THE EARLY CESSATION of growth following the removal of the pituitary has been reported in many mammals (1, 2, 3). Growth is resumed upon the administration of implants or extracts of the anterior pituitary (AP) when supplied in suitable amounts and containing the proper proportions of the necessary factors (1, 4). In fact in some normal animals, after the growth curve has begun to plateau, renewed growth can be stimulated by suitable amounts of AP extracts (5, 6). From these observations it has been assumed that the pituitary secretes one or more factors which in some way regulate somatic growth.

The ablation of certain other endocrine glands have been reported from time to time to retard growth, but in none of these is the evidence more certain than that the removal of the thyroid gland in early life slows down the rate and finally causes a complete cessation of growth (7, 8, 9). The administration of thyroid tissue or thyroxine, in suitable amounts causes a resumption of growth of thyroidectomized animals (10), and in normal animals may cause a certain increase in the growth rate (11). The effectiveness of AP extracts or implants is still a matter of controversy (12, 13).

When animals are both thyroidectomized and hypophysectomized growth stops immediately. In such animals the administration of thyroid tissue or thyroxine is without benefit. On the other hand, if pituitary extracts alone are injected there is stimulated a renewal of growth which can be further augmented by the addition of thyroxine (13).

On the basis of these observations and certain data from our laboratory (9), the theory was advanced that while a certain hormone or hormones of the AP play a primary role in the control of somatic growth, the hormone of the thyroid, thyroxine, is concerned in stimulating the rate of secretion of these AP hormones as well as influencing directly the basal metabolic rate and other somatic functions which may influence growth indirectly. Thus, when the thyroids are removed early in life, the absence of thyroxine causes the AP growth factors to gradually decrease their rate of secretion and growth gradually slows down and eventually stops.

Since the pituitary exercises control over the thyroid gland through its secretion of the thyrotropic hormone, the control of the growth rate of normal animals would be influenced in part by the factors which influence the rate of secretion of the thyrotropic hormone. Thus, within limits, the period of high thyrotropic hormone concentration in the AP (secretion?) should be a period of rapid growth and with the decline in the secretion of this hormone, growth should subside and the fattening process increase.

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In all farm animals, the rapidity of growth is an important consideration because of the continuous maintenance requirement. If the growth rate can be increased, the time to reach a given weight is decreased and the feed required for maintenance is saved. For these reasons, it seemed desirable to investigate the relation of the concentration of the thyrotropic hormone in the AP during the life cycle of various animals.

Work in this laboratory has indicated that the concentration of the thyrotropic hormone per unit of cattle pituitary tissue increased until the animals were from 4 to 10 months of age and then decreased, that male pituitaries contained greater amounts than females of similar weight, and further that dairy cattle had a higher concentration of thyrotropin than did beef cattle (14).

In swine, it was observed that during the period of rapid growth, there was a definite rise in the concentration of the thyrotropic factor in the AP and that as the growth rate slowed down the thyrotropic potency decreased. Rapid gaining pigs of the same age, breeding and environment as compared to slow gaining pigs had AP's containing 27% more thyrotropic hormone per gram of fresh tissue than did the slow gainers. Further, in strains of swine which were more rapid gainers, the concentration of thyrotropin in the AP was greater than it was in slow growers (15).

In the albino rat our study indicated that the concentration of the thyrotropic factor in the AP of both sexes increased during the period of rapid growth, with a decline as maturity was attained. However, the female pituitary in all comparisons of development contained only 62% as much hormone as the male, per gram of fresh tissue (16). It is significant that the growth rate and the maximum body weight of the female albino rat is considerably below that of the male.

The object of the present paper is to report the results of a study in continuation of our investigation of the thyrotropin concentration in the AP of growing animals. The New Zealand White rabbit represents a class of laboratory animal in which there appears to be little sex variation in either the growth rate or maximum mature weight. For that reason, the sex comparisons are believed to be of especial significance.

EXPERIMENTAL PROCEDURE

Normal New Zealand White male and female rabbits in various stages of growth were grouped as to body weight by 500 gm. intervals. The rabbits were kept in a room which did not vary in temperature more than from 50° to 70° F. They were fed a ration of mixed grain and alfalfa hay which would be considered a balanced ration for dairy cattle. As soon as an animal reached the desired weight it was killed and the pituitary, thyroids, adrenals and gonads were removed and weighed to the nearest 0.1 mg. on a precision balance. Neither the males nor females in the groups up to and including the 3,000 gm. group were used for breeding purposes. To fill out the heavier groups, animals were purchased from breeders. These females were undoubtedly multiparous and it is interesting to note the greater weight of the pituitary, adrenals and ovaries in these animals. The pituitaries were frozen immediately and held in that condition until ready for assay. The rabbits were killed during the fall, winter and early spring months.

The pituitaries in each group were macerated in a small agate mortar and suspended in distilled water. The daily amount of fluid injected in each chick was 0.1 cc. From the average weight of the chick thyroids in each group, the chick units of thyrotropic hormone were computed from the equation formulated by Bergman and Turner (17).

Assay Method.

The use of the male White Leghorn chick for the assay of the thyrotropic hormone has been described (17). Our experience during the past three years has increased our confidence in the usefulness of the male chick in the assay of this hormone. Although we have purchased our chicks from the same hatchery during the entire period, we have regularly killed from 1 to 3 chicks from every lot for controls as a check on the type of chicks received. It is interesting to note that the average weight of the normal chick thyroid varied only slightly from year to year, provided the chicks are kept under uniform temperature and light conditions (table 1). In our animal room the lights are turned on at 6 A.M. and turned off at 7 P.M.

In the early part of the year 1939, during the reconstruction of our animal room, the chicks were kept in a room in which the temperature varied from 55° to 85° F. To supply additional heat, a 100-watt light bulb was kept burning constantly over their cage. Under these conditions, the average thyroid weight of the normal chick was 4.29 mg. which was 16% greater than the average of all the normal chicks during the 3-year period.

Occasionally some of the chicks were injected with small amounts of beef muscle but no detectable weight change in the thyroids was observed. The control chicks from which the thyroid results in this paper were calculated, were hatched in 1940. The average normal thyroid weight for 127 chicks was 3.64 mg.

TABLE 1. THYROID WEIGHT OF NORMAL MALE CHICKS

Year	No. of chicks	Body wt., average	Thyroid wt., average	Standard error of mean	Room temperature
		gm.	mg.		
1938	20	55.3	3.64	±0.162	70–90° F.
1939	48	56.3	4.29	±0.173	55–85° F. Additional heat supplied with 100-watt light bulb.
1939	49	56.9	3.79	±0.197	55–85° F. No extra heat supplied
1939	37	55.2	3.86	±0.160	78–82° F.[1] No extra heat supplied to cage
1939	107	52.3	3.72	±0.083	78–82° F.[1] Extra heat supplied by electric brooder, thermostatically regulated
1940	127	52.2	3.64	±0.046	Same
1941[2]	54	53.2	3.51	±0.084	Same

[1] Thermostatically controlled room temperature.
[2] To June 1.

RESULTS

It is interesting to note that in this strain of rabbits, the females weigh as much as the males. It is possible that this is due in part to the fact that females may be kept for breeding purposes for a longer period, but it seems true that there is no distinct sex difference in either growth rate or mature body weight in the rabbit.

With increasing body weight, the average pituitary weight of the two sexes increased quite uniformly up to the 3,000 gm. group, with the females showing a slight advantage (table 2). The 3 heaviest groups of females were multiparous as compared to the virgins in the other groups. The heavier pituitary weight of these groups is believed to represent an effect of pregnancy and especially of lactation (18) (fig. 1). The average adrenal weight for each group is included.

DISCUSSION

In an attempt to interpret the meaning of the observations concerning the variation in the thyrotropic hormone content of the pituitary during growth, a number of problems arise which require discussion. There is by no means general agreement

TABLE 2. THYROTROPIC HORMONE CONTENT OF GROWING RABBITS' PITUITARY

No. of rabbits	Rabbit body wt., av.	Rabbit pit. wt., av.	Amount pit. injected per chick	No. chicks[1]	Wt. of chicks, av.	Chick thyroids wt., av.	Chick unit content					Av. thyroid wt. of rabbits	Av. adrenal wt. of rabbits
							Per gland	Per gram of pit.	Per 100 gm. body wt.	Per gland: thyroid wt.	Per gm. of pit.: thyroid		
	gm.	mg.	mg.		gm.	mg.						mg.	mg.
							Males						
19	149	4.73	24	3	51.0	4.93	0.146	30.88	0.098				
43	517	10.61	20	20	53.8	5.03	0.416	39.25	0.081	0.012	1.15	34.1	47.1
28	995	13.20	16	20	53.1	5.29	0.743	56.25	0.075	0.010	0.72	77.9	78.5
18	1522	21.36	14	20	54.6	5.09	1.239	58.00	0.081	0.013	0.62	93.8	92.2
15	2009	21.26	13	20	56.5	5.49	1.618	76.08	0.081	0.012	0.55	138.8	176.3
11	2503	26.29	13	20	54.1	5.72	2.206	83.92	0.088	0.016	0.59	141.3	242.2
13	3003	27.02	13	20	53.9	5.66	2.212	81.85	0.074	0.012	0.45	181.0	278.8
10	3483	33.34	14	20	52.4	5.62	2.493	74.79	0.072	0.010	0.31	241.2	549.2
4	4128	28.68	15	7	52.3	5.06	1.528	53.27	0.037	0.006	0.20	264.0	581.3
							Females						
18	163	4.51	24	3	54.0	4.87	0.134	29.75	0.082				
43	517	10.65	20	20	54.1	5.35	0.494	46.35	0.096	0.015	1.38	33.6	45.5
25	1013	16.35	16	20	54.3	5.59	1.056	64.56	0.104	0.014	0.85	76.3	89.9
17	1501	23.38	14	20	54.3	5.43	1.609	68.79	0.107	0.014	0.58	118.1	127.0
13	2032	23.65	13	20	54.0	5.47	1.783	75.38	0.088	0.013	0.54	139.6	203.2
11	2489	30.39	13	20	54.2	5.87	2.705	89.00	0.109	0.017	0.57	157.0	280.0
10	3054	31.22	13	20	54.5	5.12	1.981	63.46	0.065	0.012	0.39	164.2	281.5
8	3544	45.05	14	20	53.5	5.12	2.655	58.93	0.075	0.016	0.35	167.3	399.9
8	4021	41.40	15	20	53.8	4.91	2.020	48.80	0.050	0.014	0.33	149.2	531.7
8	4561	40.18	15	20	52.1	4.70	1.712	42.60	0.037	0.009	0.22	194.9	494.4

[1] Male chicks were used exclusively.

among workers in this field, especially pituitary cytologists, that a high concentration of hormone in the pituitary indicates a high rate of hormone secretion and discharge, yet it seems difficult, with few exceptions, to interpret low pituitary hormone concentration as a measure of high secretory rates. To the writers it seems more reasonable to believe that pituitary hormones which are required more or less uniformly to maintain the function of their end organs or metabolic processes would have a higher

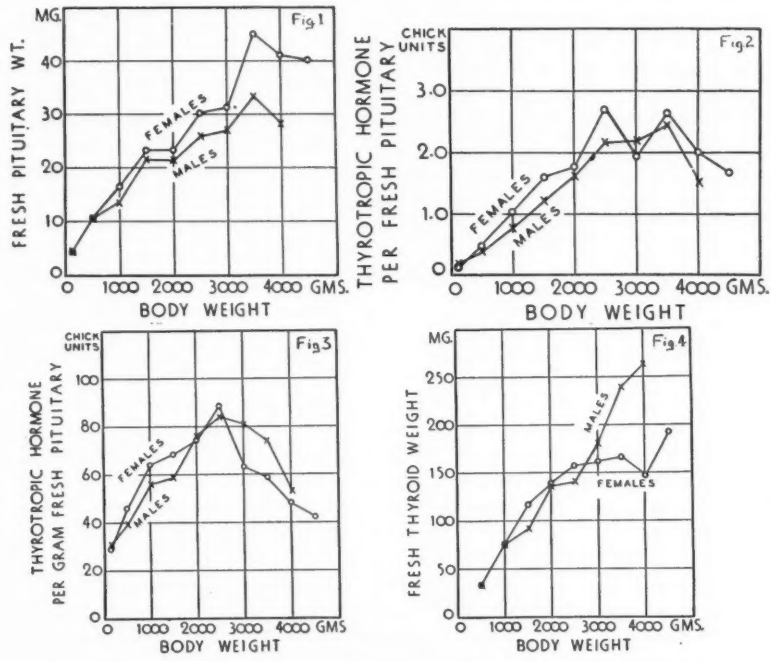

Fig. 1. AVERAGE WEIGHT INCREASE OF THE RABBIT PITUITARY DURING GROWTH. The pituitary weight of the two sexes increases uniformly up to 3,000 gm. with the females being slightly heavier. In the heaviest groups the heavier pituitary weight of the females is believed to represent the effect of pregnancy and lactation.

Fig. 2. THYROTROPIC HORMONE CONTENT OF THE PITUITARY OF THE MALE AND FEMALE NEW ZEALAND WHITE RABBIT DURING GROWTH.

Fig. 3. THYROTROPIC HORMONE CONTENT PER GRAM OF FRESH PITUITARY OF THE RABBIT DURING GROWTH. There is a gradual increase in both the male and female up to the 2,500 gm. group, then a decrease in the heavier groups. This curve might be interpreted as indicating an increased stimulation of the thyroid gland for the secretion of thyroxine during the period of rapid growth, followed by decreasing activity as fattening supersedes the growth process.

Fig. 4. THYROID WEIGHT INCREASE IN THE RABBIT IN RELATION TO GROWTH.

concentration in the pituitary during periods of higher secretion rates. In certain functions which involve a nervous integration with the pituitary, such as ovulation in the rabbit or the stimulus of the nipples in lactogenic hormone discharge, there may be coincidently a cyclic variation in the level of the hormone but it is superimposed upon a high normal level of secretion. For example, one recognizes the differences between a mammary gland which is not lactating and one secreting milk. In the case of the lactating gland, there is a cyclic variation in the amount of milk present at any given time depending upon the time elapsing since the last nursing period.

The second phase of the question in regard to the interpretation of the results obtained concerns the measure of secretory activity. Which is to be preferred, the units of hormone per gland or the units of hormone per gram of pituitary tissue? At a given body weight, would a pituitary of somewhat smaller size but with a higher concentration of hormone per unit of pituitary tissue secrete as much hormone into the blood as a larger pituitary with a lower concentration per unit of pituitary? These questions may be answered by experimental observations involving the blood and urine content of thyrotropic hormone.

On the basis of thyrotropic hormone per fresh pituitary, it will be noted that there is a gradual increase in both the male and female reaching a maximum in the 2,500 gm. group with a slight tendency to decline in the heavier groups (fig. 2). When the comparison is made upon the basis of the concentration of the hormone per gram of fresh tissue, the rising segment of the curves for both males and females are similar reaching a peak at the 2,500 gm. group and then declining with further increase in body weight (fig. 3). This curve might be interpreted as indicating an increasing stimulation of the thyroid gland for the secretion of thyroxine during the period of rapid growth of the rabbit and declining thyroid activity as fattening supersedes the growth process. The data of Belasco and Murlin (19) for the rat are interesting in this connection.

Considering the chick units of thyrotropic hormone in the AP in relation to the body weight, there is a relatively high, fairly constant, unitage per 100 gm. body weight during the period of rapid growth in both sexes, with a definite decline during the fattening period, that is in the heavier weight groups.

With increasing body weight, the thyroid weight of both sexes increased quite regularly up to the 3,000 gm. group, after which the weight in the males continued to rise but the female thyroids failed to increase much more (fig. 4). It is interesting to note that the ratio of thyrotropic hormone per pituitary to thyroid weight is relatively constant with increasing body weight whereas the ratio of concentration of thyrotropic hormone per gram of tissue to the thyroid weights is relatively high in the small animals but decreases steadily with increasing body weight. In other words, the amount of hormone which may be available to act upon the end organ (thyroid) per unit weight of that structure is maximal in the young animal when growth is most rapid and decreases in proportion as thyroid weight increases. This most interesting relation might be interpreted with more confidence if more were known about the relation of the hormone concentration to the secretory activity of varying amounts of thyroid tissue. Is a greater part of the effectiveness of thyrotropic hormone in young animals expended in stimulating cell multiplication than in stimulating thyroxine secretion? Further study will be required to answer this question.

The increase in the concentration of the thyrotropic hormone per gram of fresh pituitary tissue in the rabbit follows essentially the same pattern as that previously observed in the albino rat (16). However, while the female rat pituitary was distinctly lower in thyrotropin at every weight comparison with the males, the female rabbit pituitaries were as high if not higher in content than the pituitaries of the male rabbits of corresponding weight groups. This relation was previously reported by Chen and Van Dyke (20) who observed no difference in the total amount of thyrotropic hormone in the AP of male and female litter-mate rabbits. We believe these observations are significant in relation to the growth rate and mature weight of the two species. The female rat is considerably smaller than the male whereas the female rabbit grows to the same size as the male.

On this basis one might expect, in species of equal mature body weight, an equal concentration of thyrotropin in the two sexes. Where there are differences, a low-

ered concentration, might be expected in the smaller sex. This has been observed in the case of cattle (14), swine (15), and rats (16). It also seems reasonable to believe that an increased rate of growth might be stimulated in animals, where sex differences occur, by the injection of sufficient thyrotropin to equal that secreted by the larger sex. As an alternative, thyroid or thyroxine could be administered to supplement the normal secretion rate. Further, if it can be shown that thyroxine stimulates the rate of secretion of the AP somatic growth hormones, the role of the pituitary and thyroid in growth will be explained.

SUMMARY

Data is presented concerning the amount of thyrotropic hormone in the pituitaries of male and female White New Zealand rabbits during growth. The pituitaries were assayed in groups at 500 gm. intervals using day-old White Leghorn male chicks. The results are expressed in terms of Bergman-Turner chick units. The average amount of thyrotropic hormone in each pituitary and the concentration per gram of fresh pituitary tissue increased in each group of animals up to 2,500 gm. As the body weight increased above this, there was a decline in the thyrotropic content of the pituitaries.

Considerable significance is attached to the fact that in the rabbit the amount of hormone in the pituitaries of the two sexes is quite similar where the mature body weight of the males and females are equal, in contrast to the marked difference in the rat where the mature body weight of the two sexes differs widely.

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ASSAY OF POSTERIOR PITUITARY FACTORS WHICH CONTRACT THE LACTATING MAMMARY GLAND[1,2]

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IN 1910, Ott and Scott (1, 2) discovered that the injection of an extract of the posterior lobe of the pituitary into a lactating goat caused the contraction of the glands and the discharge of milk. These results were quickly confirmed with lactating cats and dogs (3), with dairy cattle (4) and with women (5). As is the case of other effects of the posterior pituitary extracts, there has long been a question whether the factors involved were true hormones and played a normal role in the removal of milk at nursing or milking time or whether pituitrin had only a pharmacological action on certain types of smooth muscle including those present in the mammary gland.

It has been known for many years that the injection of pituitrin causes the contraction of the mammary gland and permits the removal of milk which is otherwise unavailable (6). This phenomenon might be explained as a nervous effect, the sensory stimulation of the teats causing the motor nerves to contract the smooth muscles of the mammary gland. On the other hand, it is possible that the stimulation of the teats causes the release of a certain pituitary factor into the blood, which, passing to the mammary gland causes the contraction observed.

Some doubt was cast upon the latter theory by the observation that the removal of the posterior pituitary of the rat and dog (7, 8) did not interfere with parturition or the lactation process. However, Gomez (9) reported that lactating hypophysectomized rats could be maintained in lactation by suitable replacement therapy, but the young seemed to be unable to obtain milk present in the gland. Injections of pituitrin along with the daily 'lactation maintaining' therapy permitted the young to get the milk from the mammary glands. In a subsequent paper Gomez (10) reported that 2 to 5 U of pituitrin in equal portions 7 to 8 hours apart with the maintenance therapy permitted young to be reared until weaning age. Withdrawal of pituitrin at any time during the course of the experiment was immediately followed by a rapid loss of body weight of young, terminating in death unless pituitrin injections were promptly resumed.

Ely and Petersen (11, 12, 13) have shown that fright or the injection of adrenalin into lactating dairy cows causes cows to 'hold up' their milk. When either pitocin or pitressin was injected into the blood, the milk could be removed quickly. They reported further that a smaller quantity of pitocin induced prompt resumption of rapid milk ejection than was the case with pitressin.

While the above work is suggestive of a physiological role for the hormones of the posterior pituitary in the 'letting down' or 'ejection' of milk at milking time, it seemed desirable, if possible, to show an actual discharge of posterior pituitary hormones into the blood stream when the nipples or teats were stimulated at the time of

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milk removal. This could be done in two ways: (*a*) by showing a decrease in the amount of hormone in the pituitary after milking and (*b*) by showing an increase in the concentration of the hormone in the blood when the contraction of the mammary gland occurs.

For a number of years, the senior author has demonstrated the effect of pituitrin injected into the ear vein of the lactating rabbit to his students of milk secretion. There was a very rapid contraction of the glands with a marked dilation of the duct system. In fact the technic was used successfully as an aid in the removal of sufficient rabbit milk to analyze chemically (14). Following the separation of a pressor and an oxytocic fraction from the pituitary (15) these two fractions were tested qualitatively on the lactating rabbit. However, in such dilutions tested either fraction seemed to cause contraction of the mammary gland of the same magnitude. As the separation of the two factors was not complete, no conclusion could be drawn as to the active factor in the posterior pituitary, which influenced the mammary gland.

With renewed attention being devoted to the role of the posterior pituitary in the contraction of the mammary gland at the time of milk removal, it seemed desirable to develop an assay method for the factor concerned using the lactating mammary gland in the test. The object of the present paper is to report the details of such an assay and to indicate the reaction of posterior pituitary extracts upon the mammary gland.

DEVELOPMENT OF ASSAY METHOD

White New Zealand rabbits between the 5th and 30th days of lactation were used. The litter size varied between 4 as a minimum and 6 as a maximum. To insure maximum filling of the mammary glands, the does were removed from their young over night for a period of 15 to 18 hours before the test. The day before the animal was used for assay purposes, the hair over one or two thoracic mammary glands was shaved so as to expose the glands. It was observed that if the glands were shaved immediately before the assay, the manipulation of shaving was sufficient to cause a contraction of the glands. Whenever reshaving was necessary it was done after the assay procedure.

When the extract was to be assayed, the rabbit was gently tied on its back. If the animal struggled excessively, it was found that such animals would not respond except to very large dosage. With slight struggling, the animal could be left to lie for 5 minutes until its rate of breathing was normal and the return of color to the skin.

The extracts or posterior pituitary suspensions were injected into the large ear vein. The mammary gland is watched continuously after the injection. Within 20 to 40 seconds, if sufficient extract is administered, there is an obvious contraction of the gland with a distension of the duct system radiating from the nipple, which can be observed through the skin.

A unit of extract is defined as the minimal amount of substance, which upon intravenous injection into an unnursed lactating rabbit, will cause a definite but minimal contraction of the mammary gland within 40 seconds.

The commercial pituitrin (Parke, Davis & Co.) contained 10 international units per cc. The pitocin[3] was reported to contain 10.0 international (oxytocic) units per cc. and 0.4 pressor units. The pitressin[3] was reported to contain 10 pressor units per cc. and 0.3 oxytocic units. The pressor unit is defined as that amount of pressor activity which is associated with 1 I.U. of oxytocic activity. Pituitrin and pitocin were diluted 1:1000 with distilled water by means of 3 series of 1:10 dilutions; pitressin was diluted 1:400 for injection.

[3] The pitocin and pitressin were supplied by Parke, Davis and Co., Detroit, Mich., through the courtesy of Dr. Oliver Kamm.

EXPERIMENTAL

In the first study, using pituitrin, increasing dilutions were made until a minimal response was obtained with 0.001 I.U. of the oxytocic effect. The assay was repeated a number of times with other rabbits with essentially the same requirements for response (table 1). It is interesting to note that a unit of the posterior pituitary (PP) factor which causes contraction of the lactating mammary gland is equal to one-thousandth of the international oxytocic unit in the unfractionated pituitary extract. However, with pitocin which has been labeled to contain the same number of I.U. of oxytocic factor almost twice as many units were required to produce the same effect. In the case of pitressin 0.005 of a pressor unit was required to contract the lactating mammary gland.

TABLE 1. COMPARISON OF PITUITRIN, PITOCIN AND PITRESSIN UPON CONTRACTION OF MAMMARY GLAND

Rabbit no.	Pituitrin I.U.	Pitocin I.U.	Pitressin *Pressor* U
14	0.0010	0.0020	0.005
16	0.0015	0.0020	0.006
17	0.0010	0.0020	0.005
18	0.0010	0.0015	0.005
19	0.0010	0.0015	0.005
20	0.0010	0.0020	0.005
21	0.0010	0.0020	0.005
22	0.0010	0.0015	0.005
23	0.0010	0.0015	0.005
24	0.0010	0.0020	0.005
25	0.0010	0.0020	0.005
Average	0.00104	0.00182	0.0051

These data appear to indicate that the entire posterior pituitary contains a factor or factors which cause the contraction of the lactating mammary gland in minimum concentration. It would appear that the effect is not produced solely by the smooth muscle contracting (or oxytocic) factor because the two fractions standardized in terms of I.U. differed by almost 100% in their effectiveness on the mammary gland. On the other hand, pitressin seems to be remarkably effective considering the relatively slight contamination of the oxytocic factor. It would appear that neither pitocin nor pitressin can alone account for the phenomena under consideration. Is it possible that the factor causing the contraction of the mammary gland is another PP factor present as a contaminant in varying proportions in pitocin and pitressin? Considering the fact that the posterior pituitary secretes factors which specifically influence the contraction of the uterine muscles in one case and the smooth muscles of the vascular system in the other, it is not unreasonable to believe that it may also secrete a factor which specifically influences the contraction of the smooth muscles of the mammary apparatus.

In the preparation of cattle anterior pituitary hormones in our laboratory, the posterior lobe is separated manually as well as possible. A batch of these lobes was ground up and assayed for concentration of the mammary gland contracting factor. Seven of eight rabbits responded on 0.003 mg. of the fresh tissue. One required only 0.0025 mg. For comparison, the posterior lobes of a group of lactating rabbits were tested. A response was obtained with an average of 0.0234 mg. These preliminary data indicate that cattle posterior pituitary contains a far higher concentration of this factor than does the rabbit.

DISCUSSION

The observation first made by Ott and Scott, that extracts of the posterior lobe of the pituitary caused a contraction of the lactating mammary gland and an 'ejection' of milk has been utilized as the basis for an assay method of this factor. As smooth

muscle cells are reported around the mammary alveoli as well as in the duct tissue (16) it might be expected that the oxytocic factor would be primarily responsible for the contraction observed.

The method of assay outlined for measuring, quantitatively, the 'ejection' phenomenon of posterior pituitary extracts in lactating rabbits appeared to be extremely sensitive and very reproducible in a series of rabbits if standardized conditions were followed. In commercial pituitrin said to contain 10 I.U. of the oxytocic factors per cc. it was observed that a unit response was obtained with only 0.001 of an I.U. We were surprised to find, however, that pitocin also standardized to contain 10 I.U. per cc. and a minimal contamination of the pressor factor, required almost twice as many units of oxytocin for a response. More surprising even than this was the observation that pitressin which influences the smooth muscles of the vascular system and causes elevation in blood pressure was also very effective in causing contraction of the mammary gland even though the contamination of the extract with oxytocin was minimal.

These observations would appear to indicate either that both the oxytocic and vasopressin factors combine to influence the contraction of the mammary gland or a third factor is involved which is present in both pitocin and pitressin in varying proportions and which specifically contracts the smooth muscles of the mammary apparatus.

Studies are now in progress using this assay technic to determine if this factor in the posterior pituitary is discharged into the blood stream upon the stimulation of the nipples or teats and thus plays a normal physiological role in the 'letting down' or 'ejection' of milk at milking time.

SUMMARY

An assay method for the determination of the quantity of a factor present in the posterior lobe of the pituitary which causes the contraction of the smooth muscles of the lactating mammary gland is described. A unit of extract is defined as the minimal amount of substance which upon intravenous injection into an unnursed lactating rabbit will cause a minimal contraction of the mammary gland within 40 seconds.

In commercial pituitrin a unit response was obtained with 0.001 U of the international standard of oxytocic hormone, whereas in the pitocin reported to contain the same number of units of oxytocin 0.00182 U were required. The assay of pitressin indicated a unit effect with 0.005 U of pressor hormone.

These observations would appear to indicate either that both the oxytocic and vasopressin factors combine to influence the contraction of the mammary gland or a third factor is involved which is present in both pitocin and pitressin in varying proportions.

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BIOLOGICAL ASSAY OF THE MAMMOGENIC LOBULE-ALVEOLAR GROWTH FACTOR OF THE ANTERIOR PITUITARY[1,2]

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GOMEZ AND TURNER (1, 2) on the basis of an extensive series of experiments, postulated the presence in the anterior pituitary of two mammogenic factors, the first of which stimulated the growth of the mammary duct system and the second the lobule-alveolar system. Lewis and Turner (3) continued the study of the duct factor noting its presence in the AP of all types of cattle. Lipid extracts of the AP were shown to be rich in this factor. It was suggested that the secretion of estrogen under natural conditions and the injection of estrogen in experimental animals caused an increased secretion of Mammogen I and thus stimulated the growth of the duct system.

Since the growth of the lobule-alveolar system normally occurs during pregnancy and pseudopregnancy or upon the injection of progestin and estrogen in experimental animals, it was suggested that progestin and estrogen stimulated an increased secretion of Mammogen II by the AP which in turn was responsible for completing the growth of the mammary gland. Mixner, Lewis and Turner (4) showed that lobule-alveolar growth could be stimulated in castrate female mice by the injection of fresh AP tissue of pregnant cattle. Since the ether-soluble extract of the AP which stimulated extensive duct growth did not produce lobule-alveolar growth, it appeared that these two mammogens were separate and distinct factors.

An assay technic for the mammogen duct growth factor has been developed (5). The purpose of the present paper is to describe a technic which may be used to assay the mammogenic lobule-alveolar growth factor of the AP. As will be described later this assay method may also be used to assay for progesterone or progesterone-like substances. In this connection it may be noted that Trentin, Mixner, Lewis and Turner (6) have demonstrated that the factors in the AP which are responsible for mammary duct and lobule-alveolar growth are not progesterone or progesterone-like in nature. AP materials and extracts having mammogenic activity were assayed in the immature rabbit for progesterone activity by the sensitive McGinty technic, negative responses being secured.

PROCEDURE

Methods. The fresh pituitary material used in these experiments was prepared for injection by first grinding it through a fine meat chopper about four times. It was then mashed through an 80-mesh screen with the help of a little water.

The screened material was diluted with distilled H_2O to a concentration of about

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500 mg. of fresh tissue per cc. of H_2O, and frozen to preserve it. This stock material was diluted with distilled H_2O for injection purposes and a drop of phenol was used as a preservative.

Estrone and progesterone[3] used in these experiments were dissolved in olive oil for injection.

The technic described by Turner and Gomez (7) for the preparation and examination of the mammary glands has been followed with slight modification.

Assay animal. The virgin female white mouse weighing between 12 and 20 gm.

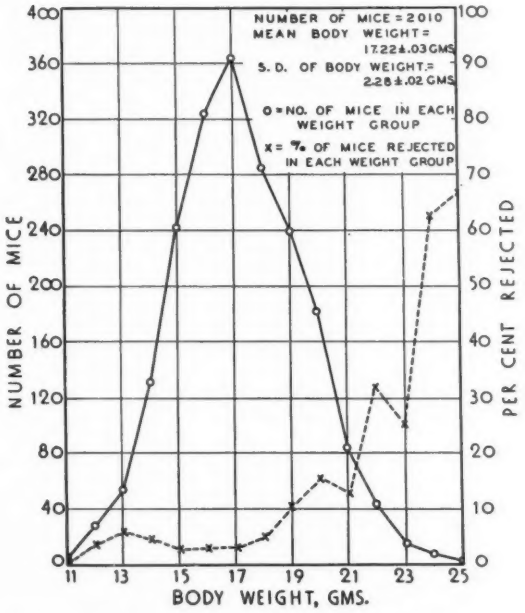

Fig. 1. Graph showing body weight and rejection of test animals. In a study of 2010 mice with body weights varying as shown, the percentage rejected because of lobule-alveolar development is fairly low (3–6%) up to 18 gm. after which time the rejection percentage increases sharply. Thus 18 gm. may be set as the upper weight limit for assay animals.

usually has a well developed smooth mammary duct system, and is a suitable animal for the assay of the lobule-alveolar growth factor. These animals are ovariectomized through an incision just lateral to the midventral abdominal line so that a check abdominal mammary gland may be taken at the same time. These check glands are fixed and stained for examination and only those animals which have a smooth duct system are kept for the assay. Figure 1 presents the relation of body weight of over two thousand mice to the rejection percentage as determined by examination of the check glands taken at the time of ovariectomy. Development beyond a smooth duct system was cause for rejection. It will be seen from the graph that if animals 18 gm. and under are selected, the number which must be discarded due to the presence of a lobule-alveolar system will be minimal.

[3] Progesterone was supplied by the Schering Corporation, Bloomfield, N. J., through the courtesy of Dr. E. Schwenk.

Assay technic. The female mice used for the assay were ovariectomized 4 or 5 days before the time they were started on injection to allow time to recover from the operation. For the assay, the animals were injected subcutaneously, daily for 10 days. On the eleventh day the animals were killed and the mammary glands prepared for examination.

Only a minimal stimulation of the lobule-alveolar system which is clearly detectable was required for a positive alveolar response. This is first evidenced by the proliferation of numerous side stubs on the main ducts and flowery ends on the short interlobular ducts (fig. 3). This stage is similar to that seen in the mammary glands of mice pregnant about four days, and is not present in the virgin female mouse which has not been pseudopregnant (fig. 2). Much greater development of the lobule-alveolar system is secured at times (fig. 4).

A mouse unit of the mammogenic lobule-alveolar growth factor may be defined as

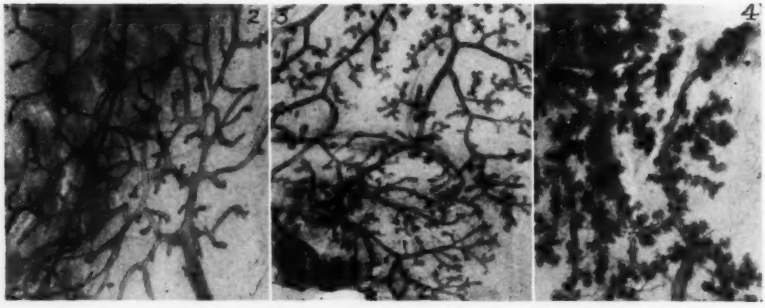

Fig. 2. SECTION OF A CHECK MAMMARY GLAND FROM A NORMAL VIRGIN, FEMALE MOUSE. Fig. 3. SECTION OF A MAMMARY GLAND OF A YOUNG SPAYED VIRGIN FEMALE MOUSE INJECTED WITH 1000γ OF PROGESTERONE AND 133 I.U. ESTRONE OVER A 10-DAY PERIOD SHOWING MINIMAL ALVEOLAR DEVELOPMENT REQUIRED FOR UNIT ASSAY RESPONSE. Fig. 4. SECTION OF A MAMMARY GLAND OF A YOUNG SPAYED VIRGIN FEMALE MOUSE INJECTED FOR 10 DAYS WITH A TOTAL OF 400 MG. OF FRESH ANTERIOR PITUITARY MATERIAL secured from non-pregnant lactating cattle showing a more advanced development of the alveolar system than is required for unit assay response (Fig. 2, 3, 4, ×9).

the total amount of pituitary material or extract required per mouse, injected subcutaneously over a period of 10 days, to obtain definite lobule-alveolar development in $50 \pm 10\%$ of 10 or more castrate, nulliparous, female mice weighing between 12 and 18 gm. Further research with this assay method may show that it may have to be modified to the extent that a small amount of estrogen be given as a basic injection to all assay animals along with the pituitary materials and extracts. Estrogen has the known effect of causing a hyperemia of genital tissues including the mammary gland and associated parts. It is suggested that the increased flow of blood to the region of the mammary gland would increase the activity of the materials which are being assayed for mammary growth, as a greater amount of the Mammogen II would be present at the site of mammary growth. Indeed this may be part of the mechanism by which estrogen enhances the activity of progesterone in causing mammary lobule-alveolar growth. The mouse unit can be applied equally well to substances like progesterone (with estrogen) whose mammary growth activity is mediated through the pituitary gland of the assay animal.

RESULTS

Six lots of fresh anterior pituitary glands secured from cattle were injected into groups of assay mice in an effort to secure graded curves of response to increasing dos-

ages of pituitary material (table 1). Lots VIII, IX, X, and XI were secured from pregnant animals; Lot XII from stock yard run of animals and Lot XIII from non-pregnant lactating animals. All injections were for a 10-day period. An examination of the '% positive' responses secured with increasing dosages of the various pituitary lots shows that strictly quantitative relationships were not obtained. At present the reasons for this are not entirely clear, but work is being conducted in an effort to further clarify the results secured. It is known that the pituitary material will decrease in

TABLE 1. LOBULE-ALVEOLAR RESPONSE OF CASTRATE FEMALE MICE TO FRESH AP FROM CATTLE INJECTED FOR 10 DAYS

Pituitary preparation	Dosage, mg.	No. of mice	Lobule-alveolar development		% Positive
			Positive	Negative	
Lot VIII	100	5	0	5	0.0
Pregnant	150	5	0	5	0.0
cattle	200	5	0	5	0.0
anterior	250	5	1	4	20.0
pituitary	300	5	1	4	20.0
	333	5	5	0	100.0
Lot IX	150	31	5	26	16.1
Pregnant	200	29	4	25	13.9
cattle	250	39	9	25	23.0
anterior	300	36	4	31	11.1
pituitary	350	24	8	16	33.0
	400	19	5	14	26.3
Lot X	100	5	0	5	0.0
Pregnant	200	20	5	15	25.0
cattle	300	19	3	16	15.5
anterior pituitary	400	6	2	4	33.0
Lot XI	100	5	1	4	20.0
Pregnant	200	5	0	5	0.0
cattle	300	25	5	20	20.0
anterior pituitary	400	6	2	4	33.0
Lot XII	100	6	1	5	16.7
Stockyard	200	5	1	4	20.0
run of	300	5	0	5	0.0
cattle	400	6	2	4	33.0
anterior pituitary					
Lot XIII	300	8	3	5	37.5
Lactating	350	6	1	5	16.7
cattle	400	8	4	4	50.0
anterior pituitary					

its alveolar stimulating potency even when stored in a frozen condition. As all injections with a given lot of material were not made at the same time, it is possible that this may be the cause of some variation. Also it is not known to what extent other factors present in the pituitary may affect the regularity of mammary growth and response.

This assay technic was further studied in an effort to check its usefulness as a quantitative assay for mammary lobule-alveolar growth promoting substances.

Turner and Frank (8) with the rabbit and Turner and Gomez (9) with the male albino mouse have reported on the mammary lobule-alveolar growth promoting properties of estrone and corporin (crude extracts). In the rabbit theelin alone or corporin

alone had little or no effect in causing lobule-alveolar growth, but the simultaneous injection of corporin and theelin caused hyperplasia of the gland lobules after 15 days similar to that found at the middle of pregnancy. In the male mouse long continued injections of estrogen also did not cause lobular growth while again simultaneous injections of estrone and corporin caused growth of lobules in normal male mice comparable to the growth observed during the first half of pregnancy in the female. These male mice were pretreated with theelin to secure growth of the duct systems.

Crystaline progesterone dissolved in olive oil was injected with a quantity of estrone which was found to be within the range for optimal mammary lobule-alveolar growth (table 2). Estrone was kept at a constant level of 133 I.U. per mouse over the 10-day period while progesterone was varied from 25 to 1500γ. From the table it may be seen that a very uniform graded response was secured with increasing dosages of progesterone. A mouse unit of progesterone according to these results is about 875γ, interpolating between 750γ with a 41.6% response and 1000γ with a 60.0% response.

Thus when a crystalline hormone of standard and unvarying potency which stimulates mammary lobule-alveolar growth in the ovariectomized mouse was assayed according to this technic, very good quantitative results were obtained.

TABLE 2. LOBULE-ALVEOLAR RESPONSE OF CASTRATE FEMALE MICE TO PROGESTERONE AND ESTRONE INJECTED FOR 10 DAYS

Reagents	Dosage	No. of mice	Lobule-alveolar development		% Positive
			Positive	Negative	
Progesterone Estrone	1500 γ 133 I.U.	3	3	0	100.0
Progesterone Estrone	1250 γ 133 I.U.	12	10	2	83.3
Progesterone Estrone	1000 γ 133 I.U.	10	6	4	60.0
Progesterone Estrone	750 γ 133 I.U.	12	5	7	41.6
Progesterone Estrone	500 γ 133 I.U.	4	1	3	25.0
Progesterone Estrone	250—25 γ 133 I.U.	16	0	16	0.0

DISCUSSION

The next phase of research in connection with Mammogen II is that of extracting the pituitary material to secure an extract which will be both potent and stable in relation to its mammogenic properties. This assay should be an excellent technic by which to check on extraction methods and on extracts.

This assay technic may have wide application in research relating to mammary gland growth. It may be used first to assay for Mammogen II, and for Mammogen II-stimulating substances from such sources as the ovaries, testes and adrenals. Secondly, it may have application as a quantitative biological assay for progesterone and progesterone-like substances, progesterone being recognized as having its typical effects on the mammary gland as well as on the uterine endometrium. Thirdly, under standardized conditions this assay could be used to determine the influence of varying conditions of nutrition, and of various hormones which may be stimulated by increasing amounts of pituitary extract, on mammary lobule-alveolar growth. For example,

increasing dosages of the thyrotropic hormone present in the pituitary extract will stimulate the secretion of larger amounts of thyroxin by the thyroid. This may in turn influence the secretion of Mammogen II by the anterior pituitary. Using progesterone and estrone as stimulators of mammary lobule-alveolar growth, it will be possible to determine the influence of varying amounts of thyroxine upon the growth of the lobule-alveolar system.

SUMMARY

A technic is reported for the assay of the mammogenic lobule-alveolar growth factor (Mammogen II) of the anterior pituitary. Other substances such as progesterone which may stimulate the secretion of the Mammogen II by the pituitary of the assay animal may also be assayed by this technic. The ovariectomized virgin female mouse is used as the assay animal. Both anterior pituitary material from cattle and progesterone were assayed. This technic may have wide application in research relating to factors affecting mammary gland growth.

A mouse unit of the mammogenic lobule-alveolar growth factor is defined as the total amount of material required per mouse, injected subcutaneously daily over a period of 10 days, to obtain definite lobule-alveolar development in $50 \pm 10\%$ of 10 or more castrate, nulliparous, female mice weighing between 12 and 18 gm.

The authors are indebted to Warren Beck for his assistance in ovariectomizing the assay animals.

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ATTEMPTS TO INDUCE MENSTRUATION BY OPERATIVE ISCHEMIA IN MONKEYS

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VARIOUS OPERATIVE PROCEDURES such as ovariectomy, excision of recent corpora lutea, aspiration of liquor folliculi from large follicles, and section of nerve supply to ovaries and uterus either (*a*) peripherally or (*b*) in the spinal cord will induce menstruation prematurely in normal monkeys. Discontinuance of hormonal stimulation of the uterus either by the withdrawal of estrogen or, after estrogenic priming, by the withdrawal of progesterone or androgen will induce menstruation in ovariectomized monkeys. These and other phases of experimental work on the endocrine mechanism of menstruation have been reviewed recently (1, 2, 3, 4).

One of the most striking changes which occurs in the endometrium during the series of events leading to either normal or experimentally induced menstruation is the vasoconstriction and ischemia lasting from 4 to 24 hours just prior to the onset of bleeding. This involves only that portion of the endometrium which is adjacent to the lumen and supplied by the coiled arteries. Although the coiled, or spiral, endometrial arteries have been known for many years, the recent work of Bartelmez (5) and Daron (6, 7) has stressed their possible physiological significance. The actual observation of their vasoconstriction in the living endometrium was first reported by Markee (8) in studies made on intraocular transplants of uterine tissue in the monkey. His further work (9) has supplied substantial evidence that this period of vasoconstriction and ischemia is an essential part of the pattern of changes associated with menstruation. He reports (9) that once vasoconstriction has begun, the superficial portion of the endometrium "receives no adequate blood supply during the remainder of the menstrual cycle." Bleeding eventually occurs as a result of the temporary opening of individual coiled arteries. The probability thus exists that during this period of endometrial ischemia, changes occur which are responsible for the extravasation of blood following relaxation of the coiled arteries.

The characteristic prolonged and profound blanching of the endometrium has thus far been produced experimentally in the castrate monkey only by withdrawal of hormonal stimulation. Hartman and Geiling (10) attempted to precipitate menstruation in monkeys by administration of the vasoconstricting drug, pituitrin. They used a slow injection method sustained over periods of several hours to insure continuity of drug action, but were unable to induce menstruation.

The present report deals with the effects of mechanically induced ischemia on the monkey uterus during various conditions of hormonal stimulation, carried out in an attempt to find some means for precipitating uterine hemorrhage other than by hor-

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[1] National Research Council Fellow.

[2] Aided by a grant from the Committee for Research in Problems of Sex of the Naitonal Research Council.

mone withdrawal. One of us had previously studied cytologically the effects of experimental ischemia on the kidney (11), and certain features of this technic seemed worthy of trial as applied to the uterus. Since the tortuosity of the cervical canal and other factors in the anatomy of the macaque uterus make it difficult to devise a method for producing ischemia limited to the endometrium alone, the present work concerns the effects resulting from interference with the circulation of the whole uterus.

EXPERIMENTAL PROCEDURE

The monkeys (*Macaca mulatta*) used in these experiments were adolescent or young mature ovariectomized females. The hormones[3] used were dissolved in sesame oil and administered intramuscularly in the amounts indicated in figure 1. The animals were examined daily and uterine bleeding recorded when evidenced by the appearance of blood externally or when observed at the external os by means of a speculum in the vagina. The color and swelling of the 'sexual skin' were also recorded as indicators of estrogenic hormone levels, but have not been included in the data presented.

The animals were anesthetized with intravenous Nembutal (25 mg. per kg.). Preliminary attempts to produce uterine ischemia by occlusion of the uterine and ovarian arteries were unsatisfactory. In only one instance did constriction of both uterine and ovarian arteries completely inhibit bleeding from an incised uterus. Clamps applied to the round and broad ligaments also proved ineffective. In the method finally adopted for the experiments here reported, the round and broad ligaments were cut (ligated if necessary) as far as the uterine arteries, and an elastic band placed around the neck of the uterus. The effectiveness of this method in withholding the uterine circulation was shown by the absence of bleeding from a uterus so treated when opened for biopsy.

The periods of arrest of the uterine circulation were varied both in duration—most of them for from 3 to 8 hours (extremes at 1 to 19 hours)—and in relation to the stage of hormone treatment which the animal was receiving (fig. 1). The periods of experimental ischemia thus were comparable in duration to the normally occurring premenstrual ischemia of from 4 to 24 hours observed by Markee. In some animals estrogen administration was continued for an interval after operation (table 1), while in others the constriction was performed on the last day of a series of injections (table 2) or on the 3rd day after discontinuance of estrogen (table 3).

Biopsy specimens and uteri removed at the termination of the experiments were preserved in Bouin's fluid and in formalin. Sections were stained with hematoxylin and eosin and by the azocarmine modification of Mallory's connective tissue stain. The uterus from one animal (not listed above) was removed aseptically, kept at 38° C., specimens fixed at intervals up to 8½ hours, and studied for evidence of progressive degenerative changes.

RESULTS

The experimental results are presented graphically (fig. 1), and in tabular form are grouped according to the stages of hormone treatment at the time of interruption of the uterine circulation (tables 1, 2, 3). The term, test, appearing in the tables refers to the attempted production of uterine bleeding by the withdrawal of estrogen following a 10-day period of administration.

In no instance did temporary interruption of the uterine circulation precipitate uterine bleeding; whereas some of the longer periods of ischemia resulted in impairment of uterine function, as evidenced by the absence of estrogen withdrawal bleeding

[3] The hormones used in these experiments were supplied by the Ciba Pharmaceutical Products, Incorporated, Summit, N. J.

during one or more subsequent tests. In experiments in which the uterus was rendered temporarily ischemic during continued estrogen treatment (table 1), bleeding was not observed so long as the hormone was continued. Except in two experiments

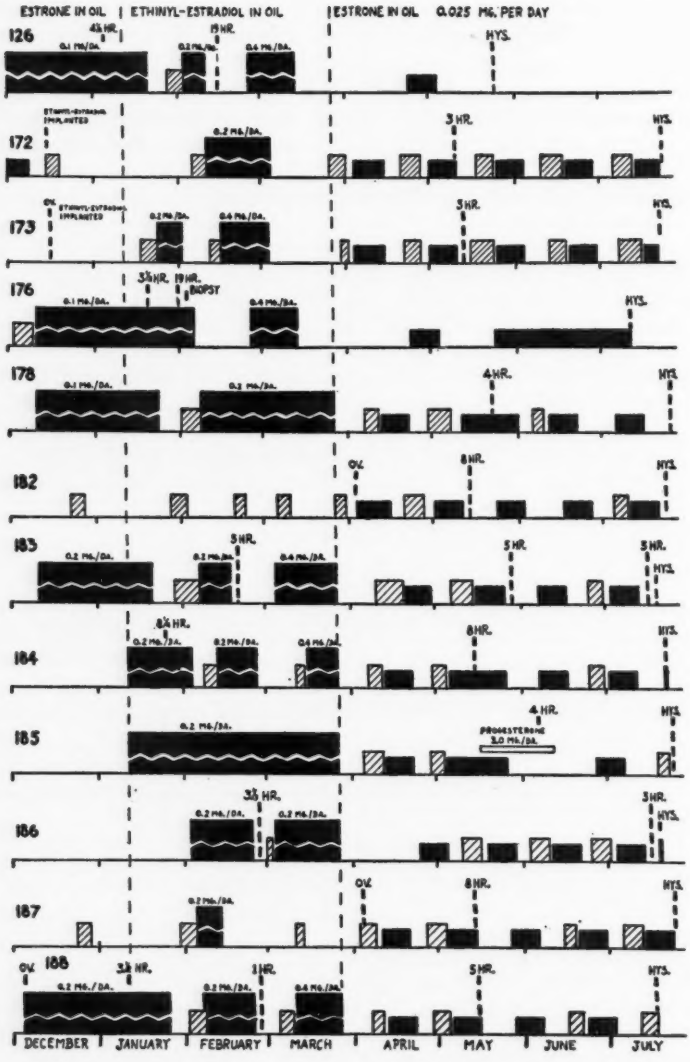

Fig. 1. HORMONAL TREATMENT, UTERINE BLEEDING, AND PERIODS OF EXPERIMENTAL ARREST OF UTERINE CIRCULATION IN OVARIECTOMIZED RHESUS MONKEYS. Estrogen administration is indicated by solid bars, uterine bleeding by cross-hatching. *Ov*, ovariectomy (animals in which ovariectomy is not indicated had been castrated previously); *Hr*, number of hours uterine circulation was interrupted; *Hys*, hysterectomy.

in which the circulation was interrupted for 8 and 19 hours respectively, a bleeding response was, however, obtained when estrogen administration was discontinued. In

the first of these two instances (*animal 184*) no bleeding occurred during 11 days after estrogen withdrawal, but absence of permanent injury to the uterus was demonstrated by the normal bleeding responses obtained in 2 subsequent tests. In *animal 176*, a biopsy removed 3 days after a 19-hour period of ischemia showed the uterus to

TABLE 1. UTERINE CIRCULATION INTERRUPTED DURING ESTROGEN ADMINISTRATION

Animal No.	Duration of ischemia	Estrogen continued after ischemia	Result during period of continued estrogen	Subsequent observations
	hr.	*days*		
176	3½	11	No bleeding	
188	3½	14	No bleeding	Bleeding on 8th day after stopping estrogen
185	4	5[1]	No bleeding	No bleeding during 15 days after stopping progesterone, normal bleeding in next test
178	4	10	No bleeding	Bleeding on 6th day after stopping estrogen, no bleeding in 2 subsequent tests
126	4½	15	No bleeding	Bleeding on 8th day after stopping estrogen
184	8	10	No bleeding	No bleeding during 11 days after stopping estrogen, normal bleeding in 2 subsequent tests
184	8¼	9	No bleeding	Bleeding on 5th day after stopping estrogen, normal bleeding in 3 subsequent tests
176	19	3	No bleeding	No further bleeding

[1] Circulation interrupted on 21st day of 26-day progesterone treatment.

TABLE 2. UTERINE CIRCULATION INTERRUPTED ON LAST DAY OF ESTROGEN TREATMENT

Animal No.	Duration of ischemia	Result	Subsequent observations
	hr.		
172	3	Bleeding on 8th day	Normal bleeding in 2 subsequent tests
188	5	No bleeding during next 12 days	Normal bleeding in 2 subsequent tests
187	8	No bleeding during next 12 days	Normal bleeding in 2 subsequent tests

TABLE 3. UTERINE CIRCULATION INTERRUPTED ON THIRD DAY AFTER STOPPING ESTROGEN TREATMENT

Animal No.	Duration of ischemia	Result	Subsequent observations
	hr.		
188	1	Bleeding on 10th day	Normal bleeding in 2 subsequent tests
173	3	Bleeding on 6th day	Normal bleeding in 2 subsequent tests
186	3	Bleeding on 6th day	Hysterectomy on 6th day
186	3½	Bleeding on 6th day	No bleeding in next test, followed by normal bleeding in 2 subsequent tests
183	5	No bleeding by 16th day	Normal bleeding in 2 subsequent tests
183	5	No bleeding by 13th day	Normal bleeding in 1 subsequent test
183	5	No bleeding by 7th day	Hysterectomy on 7th day
182	8	No bleeding by 13th day	No bleeding in next test, followed by normal bleeding on 2nd test
126	19[1]	No bleeding by 13th day	No bleeding in 2 subsequent tests

[1] Circulation interrupted on 5th day after stopping estrogen.

be congested and necrotic throughout. Later, hysterectomy after a prolonged period of estrogen administration showed an atrophic uterus with a small irregular cavity lined by epithelium resembling that of the cervix. Hysterectomy was performed on *animal 178* on the 9th day of estrogen withdrawal (fig. 1, table 1), and sections showed a compact and apparently menstruating endometrium. It is probable that blood would have appeared in the vagina had the period of observation been prolonged.

When the uterine circulation was interrupted for intervals of 3, 5 and 8 hours respectively at the end of a period of estrogen treatment (table 2), bleeding was observed in only the first instance, and then only after an 8-day latent period. No bleeding was found during a 12-day observation period following ischemias of 5 and 8 hours, although all 3 of these animals showed normal bleeding in each of 2 subsequent tests.

When uterine ischemia of from 1 to 3 1/3 hours was produced on the 3rd day after stopping estrogen administration, bleeding did not occur until 3 days later, and in one instance not until 7 days later (table 3). Normal bleeding occurred in subsequent tests in 3 of these animals, while the fourth was not further tested. When the periods of ischemia were increased to 5 and 8 hours no bleeding occurred during that cycle, although normal bleeding responses were obtained in subsequent tests. No bleeding occurred during 8 days following a 19-hour period of uterine ischemia on the 5th day after estrogen withdrawal, nor did bleeding occur in 2 subsequent tests. The uterus removed several months later was represented only by a small fibrous nodule.

DISCUSSION

In castrate monkeys the cessation of estrogen administration is typically followed by uterine bleeding within from 5 to 9 days (12, 13). The present results show no instance in which bleeding was either precipitated or its onset hastened by the experimental production of uterine ischemia. Uterine ischemia maintained for as long as 3 hours was without marked effect on either immediate or subsequent uterine function, in so far as the latter could be judged from the occurrence of estrogen withdrawal bleeding. When the intervals of ischemia were increased to from 4 to 8 hours, disturbance of uterine function was evidenced by absence of bleeding during one or more subsequent tests.

It is of interest that a bleeding response could later be obtained from uteri which had been subjected to 8-hour periods of ischemia. Sections of these uteri (*182*, *184*, *187*) showed only minor evidence of injury, the latter being more evident at the site of constriction than elsewhere. Although the extent to which regeneration may have been involved could not be estimated, the otherwise relatively normal appearance of endometrium and myometrium suggest that these structures were able to survive the prolonged ischemia.

During the normal menstrual cycle premenstrual ischemia is limited to the superficial portion of the endometrium. However, such a limitation was not possible in these experiments. Markee's consistent observation of premenstrual endometrial ischemia suggests the latter as a major cause of superficial necrosis and subsequent sloughing of tissue during menstruation. The results of the present experiment would seem to indicate that in the premenstrual uterus there are other more fundamental changes which must run their course before menstruation can occur. It might be expected that uterine ischemia would have hastened some of the retrograde changes. In some of the more severe experiments here reported, the disturbance to uterine processes was sufficient to prevent bleeding, but in no instance did experimental intervention hasten its onset.

SUMMARY

In a group of ovariectomized rhesus monkeys treated with estrogenic hormones the circulation to the body of the uterus was completely interrupted for periods varying from 1 to 8¼ hours (in 2 cases for 19 hours).

This procedure did not precipitate macroscopic uterine bleeding, nor did it hasten the onset of an expected bleeding following estrogen withdrawal.

When interruption of the uterine circulation was maintained for periods longer than 3 hours, an increasing impairment of the bleeding response to estrogen withdrawal was observed.

In 3 animals the uterus survived an 8-hour period of ischemia with subsequent return of the bleeding response to estrogen withdrawal and only slight morphological evidence of persistent damage.

It is concluded that ischemia of the whole uterus will not precipitate uterine bleeding. This does not invalidate the theory that ischemia limited to the endometrium is an essential antecedent to hemorrhage in normal and experimental menstruation.

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PROFOUND VASCULAR CHANGES INDUCED IN THE UTERUS OF THE CASTRATED RABBIT BY COMBINATIONS OF ESTRADIOL BENZOATE AND PROGESTERONE

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THE PROGESTATIONAL EFFECT of progesterone on the uterus of the castrated rabbit previously treated with estrogen is widely known (1, 2). It is also well known that estrogen can antagonize the progestational response induced by progesterone (3, 4). It is not known, however, that in castrated rabbits progesterone and estrogen combined in suitable concentrations can induce widespread vascular changes both in the myometrium and endometrium, culminating in uterine hemorrhage.

Zondek (5, 6) by using prolan A has caused uterine hemorrhage in non-castrated immature rabbits. He believed that "either from estrin or progesterone or from both a new substance is formed in the body in the interval between its production and its utilization and that this transformed substance causes bleeding."

It is the object of this paper to examine the various combinations of estradiol benzoate and progesterone which can evoke the vascular changes in the uterus of castrated rabbits. Attention will be drawn to the action of estrogen in facilitating or antagonizing the progestational effect of progesterone on the one hand, and the production of uterine hemorrhage when suitably combined with progesterone on the other. At the same time an opportunity will be taken to review briefly the claims made by Zondek for the existence of a special bleeding hormone.

MATERIAL AND METHODS

Except where otherwise stated, rabbits, castrated 12 to 14 days previously and weighing from 2 to 3 kg. were injected subcutaneously for 7 days with 0.01 mg. estradiol benzoate dissolved in 0.1 cc. sesame oil. Thereafter the rabbits were treated with combinations of estradiol benzoate and progesterone[1] for 4 days as shown in table 1. Twenty-four hours after the completion of the experiments the rabbits were all killed by a sharp blow on the back of the head. The uteri and vaginae were carefully removed from the body and rapidly fixed in Bouin's fluid or formol.

EXPERIMENTAL

Attention was first drawn to the occurrence of the vascular reactions in the rabbit's uterus, while investigating the quantitative inhibition of progesterone by estradiol. It was discovered that after pre-treatment with 0.07 mg. estradiol benzoate and then with 0.4 mg. estradiol benzoate with 20 mg. progesterone (*experiment* 1, table 1) the uteri of these rabbits showed profound changes as compared with any of those previously examined under different experimental conditions. Almost the whole thickness of the endometrium was intensively hyperemic (fig. 5). The muscle tissue had altered almost beyond recognition. The muscle fibers, especially of the inner

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[1] The estradiol benzoate and progesterone, in the form of Progynon-B, oleosum and Proluton, were supplied by Schering Corporation, Bloomfield, N.J.

layer, were widely separated by very much dilated vessels and the muscular coat resembled cavernous tissue (fig. 6). In the unfixed state the uterine horns had a livid appearance.

It was first necessary to decide whether this extraordinary vascular reaction in the uterus could be produced by the same quantity of progesterone alone or by the same or even larger quantities of estradiol benzoate alone. *Experiments* 2 to 5 were performed. Of these only the protocol of *experiments* 2 and 5 are here submitted.

Experiment 2. Castrated rabbits were given standard pre-treatment of estradiol benzoate for 7 days and thereafter a total of 4 mg. of estradiol benzoate alone was administered over 4 days. The uteri were pale and enlarged. There were 3 to 4 endometrial folds projecting into the lumen. The folds, as well as the rest of the endometrium, were lined by low cuboidal cells (fig. 1). The stroma was sodden and the major-

TABLE 1

Exp. no.	Total amount of estradiol over 4 days mg.	Total amount of progesterone over 4 days mg.	Ratio of estradiol to progesterone	Progestational reaction	Hyperemia
1	0.40	20	1:50	—	++++
2	4.00	—	—	—	—
3	0.04	—	—	—	—
4	0.40	—	—	—	—
5	—	20	—	++	—
6	0.04	20	1:500	++	—
7	2.00	20	1:10	—	++++
8	4.00	20	1:5	—	++++[2]
9	0.40	3	1:7.5	—	—
10	0.40	6	1:15	—	+±
11	0.40	12	1:30	—	++
12	4.00	1.5	1:0.37	—	+
13	4.00	3	1:0.75	—	+±
14	4.00	12	1:3	—	+++
15	0.04	0.4	1:10	—	—
16	0.04	1.5	1:37.5	—	—
17	0.04	3	1:75	+	—
18	0.04	6	1:150	++	—
19	0.04	16	1:400	+±	—
20	—	7[1]	—	+++	—

All rabbits castrated for at least 12 to 14 days and all given a total of 0.07 mg. estradiol over 7 days before injection of combinations of hormones for 4 days except *experiment* 20.

[1] No pre-treatment with estradiol benzoate.

[2] With massive necrosis of endometrium.

ity of the stromal cells contained vesicular nuclei, but a few were hyperchromatic. The stroma resembled mesenchymal tissue, except immediately underneath the surface epithelium, where it was more compact. Both the stroma and the circular muscle layers were relatively avascular (fig. 2).

The conclusion drawn from this experiment was that large amounts of estradiol up to a total of 4 mg. could not cause any noticeable hyperemia in the rabbits' uteri.

Experiment 5. After pre-treatment with estradiol benzoate, rabbits were given a total of 20 mg. progesterone alone over 4 days. The progestational reaction was less than that expected. The sides of the endometrial folds showed an increase in the number of the glands. On the tops of the folds the glands were delicate and lined by tall columnar cells, but in the fundus of the glands the cells were low columnar with relatively large round nuclei. Between the cells lining the tops of the folds there was a sprinkling of tall very narrow cells whose cytoplasm was very basophilic. Mitotic figures were numerous. Immediately deep to the glands the stroma contained a few dilated capillaries, but the number of capillaries could not be regarded as excessive.

Large quantities of progesterone alone were therefore not responsible for the vascular changes in the uterus seen in *experiment* 1. The lack of a full progestational reaction which 20 mg. should produce, we know to be due to the excessive amount of estradiol benzoate in the pre-treatment.

Since neither large quantities of estradiol benzoate nor progesterone alone could

excite the vascular reactions it was necessary to examine the effects of combinations of these hormones.

Experiment 1 showed that a total of 0.4 mg. estradiol benzoate together with 20 mg. progesterone could cause vascular changes and it seemed wise at this stage to keep the amount of progesterone constant and to vary the estradiol benzoate. Accordingly *experiments 6* to *8* were performed (table 1).

Experiment 6 (0.04 mg. estradiol benzoate plus 20 mg. progesterone). The endometrium was irregularly folded. The glands invaded the endometrium in a patchy fashion. In some cases the glands occupied the whole thickness of a fold whereas in others they occupied only a third to a half of the fold. The glands in some areas were widely dilated and looked cystic (fig. 3), the size of the cysts was accentuated by the low columnar or even cuboidal epithelium which lined the glands, unlike that which is normally found in a progestational endometrium. For comparison, however, the reaction has been regarded, arbitrarily, as a ++ response. The stroma near the tops of the folds was sodden and the cells were widely separated from each other. There were some leucocytes present. The muscle layers were pale and relatively avascular (fig. 4).

This experiment revealed that the combination of estradiol benzoate and progesterone was not optimum for initiating vascular changes. *Experiments 7* and *8* were planned to discover the effect of larger amounts of estradiol combined with the same quantity of progesterone.

Experiment 8 (4 mg. estradiol benzoate plus 20 mg. progesterone). There was an extensive degree of hyperemia in the endometrium especially in the antimesometrial pole where there was hemorrhagic necrosis associated with bleeding into the uterine cavity (fig. 7). The hyperemia in the muscles varied considerably but in the majority of cases the muscle was reduced to irregular bands scattered between the enormously dilated blood vessels (fig. 8).

Experiment 7 (2 mg. estradiol benzoate plus 20 mg. progesterone). The uteri were similar to those described in *experiment 8* except that the necrosis was patchy and not quite as extensive. In the muscular layer the vessels were not dilated to the same degree.

From *experiments 1*, *7* and *8* it follows that estradiol benzoate in amounts of 0.4 mg. or more combined with 20 mg. progesterone will excite widespread changes in the uterus, especially in the endometrium. If 0.04 mg. of estradiol benzoate or less is used with the progesterone no vascular changes are produced, but instead a glandular response is evoked.

The next series of experiments was planned to determine whether or not it was possible to produce vascular changes in the uterus with amounts of progesterone less than 20 mg. combined with 0.4 mg. of estradiol benzoate. The results of these experiments demonstrated that 3 mg. of progesterone combined with 0.4 mg. estradiol produced no obvious hyperemia (*experment* 9, table 1). A moderate degree of vasodilatation in the endometrium and less in the myometrium was produced with 6 mg. progesterone (*experiment 10*), whereas with 12 mg. (*experiment 11*) the hyperemia in the endometrium was about half as intense as that described in *experiment 1* (0.4 mg. estradiol plus 20 mg. progesterone), and there was no necrosis of the endometrium.

Since amounts of progesterone less than 20 mg. combined with 0.4 estradiol benzoate could at the most cause only some hyperemia, the estradiol benzoate was increased 10 times (4.0 mg.) and combined with varying quantities of progesterone (*experiments 12*, *13*, *14*, table 1). This series of experiments yielded some interesting information. It was shown in *experiment* 9 that 3 mg. progesterone combined with 0.4 mg. estradiol did not produce any effect on the blood vessels whereas half the amount of progesterone (1.5 mg.) together with 10 times the amount of estradiol (4 mg.) caused definite endometrial hyperemia. Three mg. progesterone combined with 4 mg. estradiol (*experiment 13*) now produced an effect on the vessels equivalent to that obtained previously with 0.4 mg. estradiol plus 6 mg. progesterone, but 3 mg. progesterone combined with 0.4 mg. estradiol as already mentioned had no noticeable influence on the hyperemia. In *experiment 14* (4 mg. estradiol benzoate plus

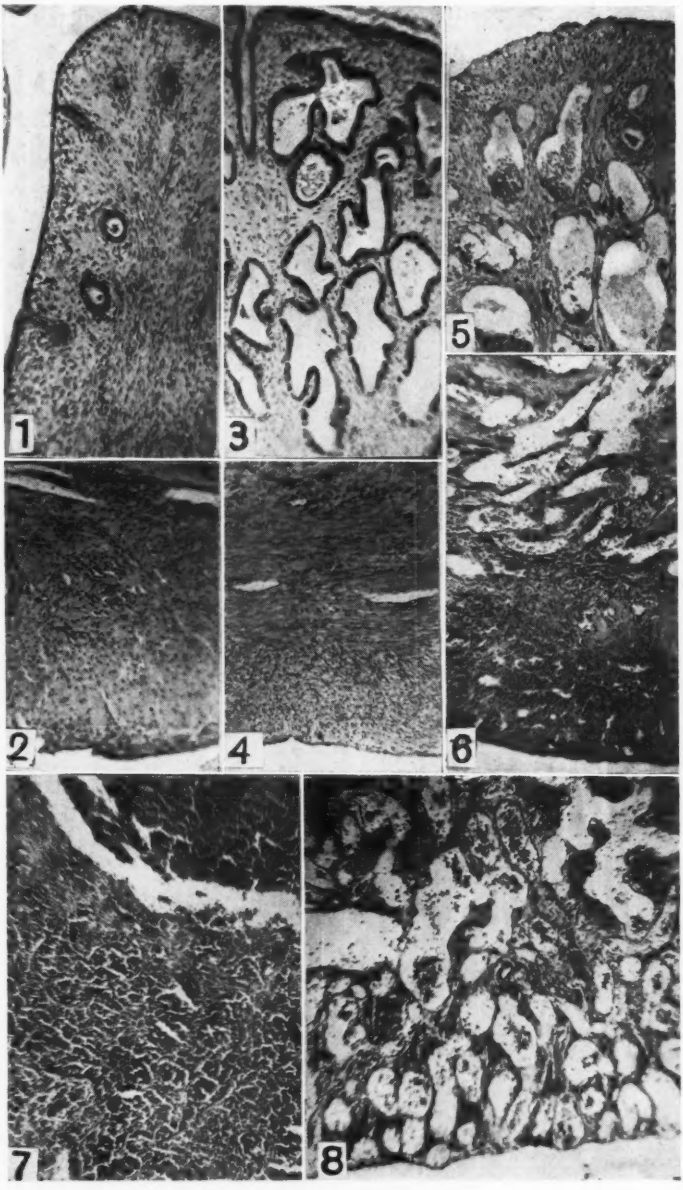

Fig. 1. Estrogenic endometrium (*experiment 2*). Fig. 2. Myometrium from same uterus as figure 1. Fig. 3. Dilated glands in endometrium (*experiment 6*). Fig. 4. Myometrium from same uterus as figure 3. Fig. 5. Extensive dilatation of vessels in the endometrium (*experiment 1*). Fig. 6. Marked dilatation of myometrium, from same uterus as figure 5. Fig. 7. Massive hemorrhagic necrosis of endometrium (*experiment 8*). Fig. 8. Extensive dilatation of vessels in the myometrium, from the same uterus as figure 7. (All photomicrographs ×70.)

12 mg. progesterone) the hyperemia was approximately a quarter less than with 0.4 mg. estradiol and 20 mg. progesterone and one and a half times more than after 0.4 mg. estradiol benzoate and 12 mg. progesterone (*experiment 11*).

In none of the *experiments 12* to *14* was the surface epithelium of the uterus destroyed by hemorrhage and for that matter the blood was confined to the dilated blood vessels in the endometrium. The increased quantity of progesterone seemingly enhanced or 'stepped up' the action of the progesterone as far as its effect on the uterine vessels was concerned (table 1).

There was one more question to be answered: did the hyperemia in the uterus depend on the ratio between the estradiol benzoate and progesterone employed, or did it depend on the absolute amounts of these hormones? The answer was partially implicit in the findings of previous experiments (table 1), but *experiments 15* to *19* were performed to settle this problem definitely. From table 1 it is clear that the vascular reaction is not as dependent on the direct ratio between the two hormones but it does depend on the absolute quantities used, since in *experiment 15* the ratio of estradiol benzoate to progesterone is 1:10 and there was no effect on the blood whereas in *experiment 7* the ratio is the same as in *experiment 15* yet there is extreme hyperemia of the uterus.

DISCUSSION

Hyperemia of the uterus has been observed both in normal and experimentally treated rabbits. Estrogen is said to cause hyperemia in the rabbits' uterus within half an hour of its administration and this subsides several hours later (7). Greep (8) injected estrogen intravenously into hypophysectomized infantile rabbits and found that bleeding took place into the submucosal tissues 6 days later. He expressed the opinion that this hemorrhage was associated with involution of the uterus after estrogen stimulation. Markee (9, 10) by using the ocular transplant technic has demonstrated that maximum dilatation of the uterine vessels occurs several hours after ovulation and persists until the 10th day after a sterile copulation. Similarly it is generally known that the rabbit's uterus in early pregnancy is very hyperemic. Reynolds (7) was of the opinion that free blastocysts act as an inflammatory agent upon the endometrium giving rise to pronounced reactive hyperemia.

Zondek (6) stated that in immature non-castrated rabbits treated for 5 days with 100 R.U. of gonadotropic hormone (prolan A) he was able to produce hemorrhages into the uterine mucosa with an escape of blood into the uterine cavity. Since he was unable to produce any uterine hemorrhages with prolan A in castrated animals he assumed that the gonadotropic hormone mobilizes a bleeding substance in the ovary.

While there is very little doubt that the withdrawal of estrogen will cause patchy endometrial hemorrhages in rabbits (8) there seems equally no doubt that the giving of large quantities of estradiol benzoate will not cause any widespread vascular reactions, provided, of course, that the uteri are examined while still under the influence of estrogen (*experiments 2, 3, 4*). On the other hand estradiol benzoate plus progesterone in adequate amounts will not only lead to a great vasodilatation of the vessles in the uterus but in certain cases will actually lead to uterine hemorrhage (table 1). Progesterone alone in castrates does cause a slight endometrial hyperemia but nothing like that observed when estradiol benzoate and progesterone are suitably combined. In pseudopregnancy and in pregnancy the hyperemia of the uterus is most probably due to the presence of both estrogen and progesterone but in combinations not yet determined. The hyperemia in these instances is not so intense as produced experimentally and the glands undergo a marked progestational metamorphosis. In the experimentally produced bleeding the glands undergo no obvious change and bleeding takes place from a 'proliferative' endometrium.

It may be that, in the experiments performed by Zondek, the ovary of immature non-castrated rabbits liberated a substance responsible for the uterine bleeding. It is quite clear, moreoever, that suitable combinations of estradiol benzoate and progesterone in mature castrated rabbits will also produce uterine hemorrhage. Uterine bleeding in the rabbit, therefore, can be produced in the absence of ovaries and in the presence of estradiol benzoate and progesterone. It is possible that the giving of prolan A to immature rabbits leads to the production of large quantities of estrogen and progestogen in concentrations sufficient to evoke uterine hemorrhage. In a later communication, however, Zondek (5) revised his opinion and suggested that a transformed substance of progesterone might cause bleeding whereas in the same paper he suggests still further that this transformed substance might arise from estrogen, progestogen or from both. It still remains to be established whether such a 'transformed substance' does in fact exist and that this substance is able to precipitate bleeding.

Two facts of particular importance emerge from this investigation, namely, that estradiol benzoate steps up the action of progesterone and that these two hormones in certain definite combinations exert a profound effect on the vascular mechanism of the rabbit's uterus.

It has been known for some time that estrogen facilitates the action of progesterone (11). Similarly it has been established that estrogen in certain concentrations will inhibit progesterone (3, 4, 12, 13). Progesterone in castrated rabbits can alone produce a progestational response (14) but apparently in much greater amounts than if the uterus were previously treated with very small quantities of estrogen. In this respect, then, optimum quantities of estrogen facilitate the progestational action of progesterone and make it, so to speak, more usable by the body. In the same way, moreover, we find that large quantities of estrogen, although they inhibit the progestational effect of progesterone, nevertheless enhance the hyperemic action of progesterone. Thus we see that although 0.4 mg. estradiol benzoate combined with 3 mg. progesterone will not cause any appreciable effect on the hyperemia of the uterus (*experiment* 9, table 1), 3 mg. progesterone combined with 4 mg. estradiol benzoate (*experiment* 13) causes an effect which hitherto could be achieved only by 6 mg. progesterone combined with 0.4 mg. estradiol (*experiment* 10). As the estradiol is increased less progesterone is necessary to cause the hyperemia. This is particularly obvious when comparing the results of *experiments* 9 and 12. In the former 0.4 mg. estradiol and 3 mg. progesterone had no noticeable effect on the hyperemia, whereas in the latter 10 times the amount of estradiol and half the quantity of progesterone produced a distinct hyperemia.

It seems, therefore, that the amounts of estrogen and progestogen required to produce hyperemia are inversely proportional to one another, i.e., if relatively small quantities of estradiol are employed then more progesterone is required and vice versa. It is necessary to re-emphasize the fact that so far as is known from our experiments the absolute amount of estradiol is a most decisive factor and that below certain absolute levels it is difficult to produce hyperemia even in the presence of large quantities of progesterone.

Further, the direct ratio between the amounts of estradiol benzoate and progesterone gives no indication of the efficiency of the combined hormones in producing hyperemia in the rabbit's uterus (compare *experiments* 7, 9, 15).

It is obvious from the figures that the hemorrhage induced in the endometrium takes place in the proliferative phase. In early pregnancy and in pseudopregnancy the hyperemia in the uterus is associated with a progestational endometrium. From the superb work of Browne and Venning (15) and Smith and Smith (14) it is becoming increasingly recognized that during pregnancy not only is there an abundant secretion of progesterone but also of estrogens. It seems possible that with suitable care in regu-

lating the balance between estrogen and progesterone a typical 'pregnancy' hyperemia can be induced in the castrate uterus.

The second fact to be considered is the production of hyperemia and even severe hemorrhage from the uterus by combinations of estradiol and progesterone. Estradiol alone when administered in the fashion described above has no noticeable effect on blood vessels; progesterone alone as also mentioned acts chiefly on the glands. Estradiol in certain concentrations inhibits the progestational effect of progesterone and when estradiol and progesterone are administered they lead to the appearance of a new set of reactions in the uterus, which involve almost exclusively the vascular mechanism leading eventually to uterine bleeding. This phenomenon as set forth in this paper has up to the present not been ascribed to these hormones and it may be of considerable practical importance.

If these results can be confirmed in primates then we may perhaps obtain a new slant on the etiology of accidental hemorrhage and perhaps even of eclampsia. Smith and Smith have shown the close interrelation between the metabolism of the estrogens and progestogen during the menstrual cycle and pregnancy, and that this metabolism is disturbed in the pre-eclamptic state. Treatment with either estrogen or progestogen may be fraught with some danger as the administration of large quantities of estrogen in the presence of progesterone or vice versa may produce a balance adequate to excite hemorrhage.

SUMMARY

It has been shown that estradiol benzoate and progesterone when suitably combined and injected into castrated rabbits will cause widespread vascular changes in the endometrium and myometrium including uterine hemorrhage.

Attention has also been drawn to the fact that in castrated rabbits estrogen when suitably combined with progesterone will cause a progestational reaction; or it will inhibit the progestational effect of progesterone or again it will lead to profound vascular changes in the uterus. These reactions depend exclusively on the absolute amounts of estradiol and progesterone employed.

It has been demonstrated that within limits the amounts of estradiol and progesterone required to produce vascular changes in the uterus are inversely proportional to one another.

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OBSERVATIONS ON HORMONE WITHDRAWAL BLEEDING IN CASTRATED MACAQUES[1]

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THE MACACUS RHESUS MONKEY has become important in the study of the mechanism of menstruation because these animals periodically bleed at intervals which approximate those in the human (1, 2, 3). The normal occurrence of anovulatory as well as ovulatory menstrual cycles was first definitely established in these animals (3–7).

When the ovaries of normal macaques have been removed, the ovulatory type of menstrual cycle may be simulated by the injection of estrogenic substances during a 'priming' period, which is followed by treatment with progesterone (8–14). With the cessation of this hormone treatment, bleeding occurs (10, 14–17). The anovulatory type of cycle may be produced by treatment with estrogen alone (5, 18–30). In some instances bleeding may occur during the course of the injections (5, 9, 19, 26, 28, 31). Bleeding always follows the withdrawal of an appropriate estrogen stimulus (5, 9, 18–30).

The period of time elapsing between the cessation of treatment of castrated macaques and the appearance of the experimentally induced bleeding is known as the latent period. The latent period for bleeding following estrogen withdrawal in the macaque has been found to vary from 5 to 16 days (5, 17–21, 24, 29, 32–34). The latent period for bleeding following cessation of estrogen-progesterone treatment is shorter and has a more narrow range of 1 to 4 days (5, 9, 13, 16, 17, 35, 36).

The bleeding time following estrogen withdrawal ranges from 2 to 9 days, according to the majority of the reports (5, 19, 21, 22, 24, 29, 32, 33, 34). Bleeding after treatment with estrogen-progesterone lasts 2 to 9 days (5, 17).

In the human the normal bleeding time has been defined as having a range of 2 to 7 days with intervals as short as 24 and as long as 33 days. A modification of the established interval and period of flow in the human leads to menstrual disorders of different types. The most common form of modified menstrual phenomena is a shortening of the interval accompanied by an increase in the amount and duration of the menstrual flow. One theory of the pathogenesis of this condition (37) postulates that a partial failure of the function of the corpus luteum is associated with shortened intervals and prolongation of the flow; that progressive modification of the function of the corpus luteum is associated with the development of irregular cycles and bleeding periods of various length and profuseness; and that complete loss of the function of the corpus luteum in such cases is associated with periods of amenorrhea which may alternate with periods of prolonged bleeding. Often a hyperplasia of the endometrium occurs under these conditions (38).

It has recently been pointed out (39) that little has been done in the experimental labora-

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tory with macaques to simulate the conditions of pathologic uterine bleeding observed in the human. Periods of bleeding in the castrated macaque which have lasted longer than 7 days after estrogen withdrawal have been reported twice (19, 22). The duration of the bleeding in these instances was 8 and 9 days. One instance of a bleeding period which was longer than 7 days after treatment with estrogen-progesterone has been found in the literature (17). Hyperplasia of the endometrium has been produced in the macaque by the use of estrogenic material, but the pathologic uterine bleeding commonly associated with the spontaneous condition did not occur (40–43).

The data reported in this paper on the latent period and bleeding time in castrated macaques following cessation of treatment with estrogen alone and with estrogen-progesterone, were obtained during a series of experiments designed to yield endometrial biopsy material on the first day of hormone withdrawal bleeding. Nearly 50% of the animals in this study bled for periods of time longer than seven days.

MATERIAL

Ten mature castrated macaques were employed in this study. Twenty-one experimental bleeding periods were induced in these animals as the result of ovarian

TABLE 1. ESTROGEN WITHDRAWAL BLEEDING IN CASTRATED MACAQUES. DURATION: 7 DAYS AND LESS

Animal No. and date	Current Experiment				Preceding Experiment. Treatment
	Treatment: 100 R.U. estrogen daily	Latent period	Bleeding time	No. prev. biopsies	
	days	*days*	*days*		
M22, 12/14	14	10	6	0	
M23, 1/2	14	9	7	0	
M26, 2/1	14	6	4	1	Estrogen-progesterone
M14, 12/7	21	6	4	2	Estr. 3 mo. massive[1]
M15, 12/16	28	8	4	1	Estr. 3 mo. massive[1]
M16, 12/15	28	6	5	1	Estr. 3 mo. massive[1]

[1] These 3 animals had been employed in an experiment designed to produce endometrial hyperplasia. *M15* and *M16* had received 2,500 R.U. of estradiol benzoate daily for a period of 3 months. *M14* had received 5,000 R.U. of estrogen daily for 3 months. Hyperplasia was not found in the endometrium of any of these animals. Experiment had terminated 8/3/40.

hormone treatment and its withdrawal. The materials employed were estradiol benzoate in oil,[2] and progesterone.[2] The dosage level of estrogen employed in these studies was chosen because it had been suggested[3] that 100 R.U., estrogen daily for a period of 14 days would produce in the macaque an endometrium characteristic of the proliferative phase of the normal cycle of the macaque.

Twelve of the bleeding periods were produced in animals treated with 100 R.U. estrogen daily for 14 days (4 animals), 21 days (2 animals), 28 days (2 animals), 35 days (2 animals), and 42 days (2 animals) (tables 1 and 2).

Seven periods of bleeding were induced in animals which were primed for 14 days with 100 R.U. estrogen daily, and then were treated with the same level of estrogen in addition to progesterone. The dosage of progesterone was 1 RB.U. daily for 7 days (2 animals), and 1 RB.U. daily for 14 days (2 animals). In 3 animals the dosage of progesterone was 2 RB.U. daily for 14 days. It should be remembered that the level

[2] Estradiol benzoate, in the form of Progynon-B, and progesterone, in the form of Proluton were generously supplied by Dr. Erwin Schwenk of Schering Corporation, Bloomfield, N. J.

[3] Personal communication, Dr. Carl Hartman.

TABLE 2. ESTROGEN WITHDRAWAL BLEEDING IN CASTRATED MACAQUES. DURATION: MORE THAN 7 DAYS

Animal No. and date	Current Experiment				Preceding Experiment. Treatment
	Treatment: 100 R.U. estrogen daily	Latent period	Bleeding time	No. prev. biopsies	
	days	*days*	*days*		
M27, 2/20	14	9	8	1	Estrogen, 21 days
M27, 1/7	21	7	8	0	
M16, 2/17	35	7	8	2	Estrogen, 28 days
M15, 2/16	35	7	9	2	Estrogen, 28 days
M28, 2/3	42	7	8	0	
M25, 2/3	42	7	10	0	

of estrogen was maintained at 100 R.U. daily during the period of treatment with progesterone in all of these animals (tables 3 and 4).

Bleeding occurred in two animals in which the priming period was 14 days, and the level of estrogen totaled 2700 R.U. administered as follows: 100 R.U. estrogen daily for 10 days; 200 R.U. on the 11th day, and 500 R.U. on days 12, 13 and 14. On

TABLE 3. ESTROGEN-PROGESTERONE WITHDRAWAL BLEEDING IN CASTRATED MACAQUES: DURATION: 7 DAYS AND LESS

Animal No. and date	Current Experiment						Preceding Experiment. Treatment
	14-day priming period, estrogen	Estrogen-progesterone		Latent period	Bleeding time	No. prev. biopsies	
		daily dosage	*days*	*days*	*days*		
M14, 1/24	100 R.U./dy.	100 R.U.; 1 RB.U.	7	5	6	3	Estr. 21 days
M22, 3/30	100 R.U./dy.	100 R.U.; 1 RB.U.	14	4	7	2	Estr.-prog.
M22, 2/3	100 R.U./dy.	100 R.U.; 2 RB.U.	14	5	3	1	Estr. 14 days
M26, 12/19	2700 R.U. total	15 R.U.; 1, 2, 3 RB.U.	14	4	3	0	
M14, 3/20	2700 R.U. total	15 R.U.; 1, 2, 3 RB.U.	14	4	2	4	Estr.-prog.

day 15 the dosage of estrogen was dropped to 15 R.U. daily and was maintained at that level through the 28th day, while 1 RB.U. progesterone was administered simultaneously on day 15, 2 RB.U. on day 16 and 3 RB.U. on day 17 and daily thereafter at that level through day 28 (table 3).

The animals were examined twice daily during the latent period, and the appearance of blood at the external vaginal orifice was considered evidence of the onset of

TABLE 4. ESTROGEN-PROGESTERONE WITHDRAWAL BLEEDING IN CASTRATED MACAQUES. DURATION: MORE THAN 7 DAYS

Animal No. and date	Current Experiment						Preceding Experiment. Treatment
	Priming period with estrogen		Estrogen-progesterone		Latent period	Bleeding time	No. prev. biopsies	
	dosage	*days*	*daily dosage*	*days*	*days*	*days*		
M25, 3/20	100 R.U.	14	100 R.U.; 1 RB.U.	7	3	12	1	Estr. 42 days
M26, 3/27	100 R.U.	14	100 R.U.; 1 RB.U.	14	3	16	2	Estr. 14 days
M28, 3/28	100 R.U.	14	100 R.U.; 2 RB.U.	14	4	26	1	Estr. 42 days
M23, 2/23	100 R.U.	14	100 R.U.; 2 RB.U.	14	4	30	1	Estr. 14 days

bleeding. The daily examination of the animals continued until a few days after the cessation of bleeding. When the animals were to be employed in subsequent experiments, the next series of injections was not begun until 28 days had elapsed since the last injection of the preceding experiment.

OBSERVATIONS

The data on the latent period and bleeding time have been grouped in tables 1 to 4 on the basis of the type of hormone treatment and the length of the bleeding time. Operative data are included in the column 'number of previous biopsies' and the hormone treatment in the previous as well as in the current experiment is given in the tables for the sake of completeness in the report.

Bleeding in castrated macaques following the cessation of estrogen injections

Bleeding periods of 7 days and less (table 1). There were 6 animals in this group, none of which were treated longer than 28 days with the uniform dosage of 100 R.U. of estrogen each day. The latent period varied from 6 to 10 days with an average of 7.5 days. The two longest latent periods were in 2 animals which had not been previously biopsied. The bleeding time ranged from 4 to 7 days with an average of 5 days for the group. The 2 animals which had not been previously biopsied and which had had the longest latent period bled for 6 and 7 days respectively.

Bleeding periods of more than 7 days (table 2). There were 6 bleeding periods represented in this group. The dosage of estrogen was 100 R.U. daily for 14 days (1 animal), 21 days (1 animal), 35 days (2 animals), and 42 days (2 animals). The latent period was 7 days in 5 instances and 9 days in the sixth. The bleeding time was 8, 9 and 10 days with an average of 8.5 days. The average duration of bleeding for the first group of animals was 5 days.

Bleeding in castrated macaques following the cessation of estrogen-progesterone injections

Bleeding periods of 7 days and less (table 3). There were 5 bleeding periods in this group. Two of the animals had had the estrogen level lowered during the period of treatment with progesterone. In the other 3 instances the level of estrogen was maintained at 100 R.U. daily throughout the period of progesterone treatment. The latent period was 4 and 5 days for all of these animals, with an average of 4.4 days. This was shorter than the average of 7 days for the latent period following the withdrawal of estrogen treatment. The bleeding time varied from 2 to 7 days, with an average of 4.2 days. Two animals bled for 6 and 7 days respectively, and each had had 3 and 2 previous biopsies, while the level of estrogen had been maintained at 100 R.U. daily throughout the period of progesterone treatment.

Bleeding periods of more than 7 days (table 4). In all 4 of the animals in this group, the level of estrogen had been maintained at 100 R.U. daily throughout the period of progesterone treatment. One animal received progesterone for 7 days, (1 RB.U. daily), a second had received the same daily dose of progesterone but for 14 instead of 7 days, while two had received 2 RB.U. progesterone daily for 14 days. The latent, period was 3 and 4 days for this group of animals. This was the shortest latent period recorded for any group in this series of experiments. The bleeding time was 12, 16 26 and 30 days respectively. The animal bleeding the shortest period, 12 days, had received 1 RB.U. progesterone for 7 days. The animal bleeding 16 days had received

the same amount of progesterone for 14 days. The 2 animals bleeding 26 and 30 days had received identical treatment, 2 RB.U. of progesterone daily for 14 days. It is to be remembered that the level of estrogen was 100 R.U. daily throughout the period of treatment with progesterone.

SUMMARY

A total of twenty-one bleeding periods followed withdrawal of treatment with estrogen alone and with estrogen-progesterone in castrated macaques. Eleven of the bleeding periods were 7 days or less in length, and 10 of them were longer than 7 days. The average bleeding time in those animals which bled longer than 7 days after the withdrawal of estrogen was 8.3 days, the longest period being 10 days. In those animals bleeding longer than 7 days after the withdrawal of estrogen-progesterone, the average duration of the flow was 21 days, the shortest period being 12, the longest 30 days. All of the animals in the latter group had received the same dosage of estrogen during the period of treatment with progesterone that they had received during the period of estrogen priming.

The prolongation of bleeding after treatment with estrogen-progesterone appeared to be related somewhat to the balance between the two hormones during the period of simultaneous treatment, and to the amount of estrogen administered during the priming period. The period of bleeding for 12 days occurred after the smallest amount of progesterone for the shortest period of time (1 RB.U. progesterone for 7 days). The longest periods of bleeding occurred in the two animals which received 2 RB.U. progesterone daily for 14 days.

No consistent relationship between the number of previous biopsies and the length of the bleeding time, or between the treatment in the preceding experiment and the bleeding time in the current experiment, was apparent in this series of observations.

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INFLUENCE OF LARGE DOSES OF ESTROGENS ON THE BLOOD PICTURE OF RHESUS MONKEYS (*Macaca mulatta*)

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THE PRESENT STUDY was made on rhesus monkeys of the Carnegie colony in Baltimore, which had been receiving huge quantities of estrogens over long periods of time. This work seemed all the more interesting because the rhesus monkey has proved to be far more tolerant of estrogens than the dog (4) or the mouse.

The animals were kept on a mixed diet (1). Blood was drawn at 9 A.M. from the femoral vein, for all determinations. Red and white blood cells were counted in the improved Neubauer chamber. Hemoglobin was determined with the Newcomer hemoglobinometer. Differential counts were made on smears stained with Wright's stain, and at least two hundred cells were counted. For the determination of reticulocytes at least 500 cells were counted. Cell volume was determined with Wintrobe's (2) hematocrit tubes.

Counts determined in 4 untreated animals (8 observations) showed values similar to those established by Shukers, Langston and Day (3) (table 1). Estrogens were administered to the animals as follows.

No. 604. Five mg. (50,000 I.U.) of estradiol dipropionate was injected weekly from Jan. 4 to March 31, total 65 mg. From March 13 to April 2, 1 mg. of progesterone (Proluton, Schering) was injected daily, except on two Sundays, total 18 mg. Blood samples were taken as shown in table 1.

No. 606 was treated with daily doses of 1 mg. estradiol dipropionate (Schering) from Jan. 4 to April 3 (except on Sundays), a total of 73 mg. One mg. of progesterone per day was injected from March 13 to 23, then 25 mg. of testosterone propionate from March 24 to April 1 (except Sundays). Blood samples were taken as shown in table 1.

The dosage of estrogens employed in these 2 animals were small compared with the following and were complicated by the use of progesterone, which latter condition was reflected in the blood picture.

No. G 21 (wt. 3470 gm.) received two 3-mg. pellets of estrone placed subcutaneously and 3 injections of 10,000 I.U. of Amniotin (Squibb) weekly from Nov. 11, 1938 to Feb. 10, 1939. The number of pellets was then doubled until April 14, after which the addition of pellets was discontinued and the Amniotin dosage doubled until June 30, when administration of estrogens were discontinued for the summer. From Oct. 23, 1939 on 2 estrone pellets were given weekly. The 2 samples which were analyzed, it is seen in table 1, were taken one year apart.

No. G 22 (wt. 3950 gm) was similarly treated up to the reading shown in table 1. By Jan. 11, 1939, she had received 240,000 I.U. of Amniotin and 18 pellets of estrone.

No. G 23 (wt. 2890 gm.) was oöphorectomized before the experiment began. Up to the time the readings shown in table 1 were made she was treated in the same manner as the preceding.

No. G 24 (wt. 3970 gm.) treated in the same manner as *No. G 21*.

No. G 25 (wt. 2670 gm.) oöphorectomized before the experiment began and then treated in the same manner as *No. G 21*.

No. G 28 (wt. 2630 gm.) oöphorectomized before the experiment began. On April 11, 1939 this female received 800 mg. of estradiol crystals under the skin and after that five 3-mg. pellets of estradiol

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TABLE 1

Monkey no., sex and date	R.B.C. millions per cm.	Hemoglob. gm./100 cc.	Vol. packed red blood cells cc./100 cc.	Mean corp. vol. cu. μ	Mean corp. hemoglob. γ	Mean corp. Hb. concentr. %	Reticulocytes %	W.B.C. thousands	Myelocytes %	Juvenil %	Segm. N. %	Lymphocytes %	Eosinophils %	Basophils %	Monocytes %	Platelets thousands
Means[1]	5.2	12.2	40.0				0.6	15.1			36.0	59.0	0.5	0.5	3.0	475
Means[2]	5.32	12.2	39.9	75	23	31	0.7	14.4	—	0.5	52.0	43.0	1.0	0.5	3.0	332
604♂[3]																
1/10	5.03	12.00	40.0	79	24	30	0.5	8.8	—	7	49	40	1.5	—	2.5	422
1/31	4.80	11.38	37.0	77	24	30	0.6	14.2	—	1	51.5	35.5	6.0	—	6.0	566
2/14	4.09	10.00	31.0	76	27	32	1.6	21.6	0.5	5.5	50.5	41.5	0.5	—	1.5	230
3/7	3.86	8.70	30.0	78	23	29	0.3	10.7	—	—	60	37	1.0	—	2.0	288
3/14	2.97	7.84	29.0	97	26	27	0.1	8.6	—	—	47	49	3.0	—	1.0	438
3/23	3.70	10.50	29.0	78	28	36	3.4	11.2	—	—	51	40	6.0	—	3.0	504
4/3	3.64	10.00	27.5	77	28	36	0.2	14.1	—	1	74	24	—	0.5	—	620
606♂[3]																
1/10	4.90	11.80	39.0	79	24	31	0.6	14.5	—	3	83	12	—	—	2.0	380
1/31	4.45	11.17	35.0	79	25	32	0.6	21.8	0.5	5	73	17	1.5	0.5	2.5	648
2/14	4.30	10.50	35.0	82	24	30	1.2	7.6	—	—	74	25	1.0	—	—	552
2/28	3.50	9.00	28.0	80	25	32	0.9	6.3	—	—	69	30	—	—	1.0	312
3/14	3.56	9.00	31.8	90	25	28	0.0	16.5	—	—	72	18	4.0	4.0	2.0	580
3/23	4.11	10.80	30.0	73	26	36	3.6	18.9	—	1	76	21	1.0	—	1.0	600
4/3	4.19	10.40	33.5	80	25	31	2.4	17.9	—	1	75	22	—	—	2.0	544
G21♀																
1/18/39	4.86	11.80	39.0	79	24	31	0.8	10.2	—	3	61	29	2.0	0.5	4.5	406
1/25/40	5.09	11.48	39.5	78	23	29	1.4	7.9	1	2	54	32	2.0	2.0	7.0	230
G22♀																
1/11/39	5.20	13.10	40.0	77	25	33	0.1	15.8	—	5.5	30.5	58.5	0.75	—	4.75	358
G23♀																
1/18/39	4.60	11.80	31.0	68	26	38	1.0	8.2	—	4	73	21.5	—	—	1.5	108
G24♀																
1/25/40	4.38	10.77	35.5	80	25	30	0.6	10.5	—	—	54	43	1.0	1.0	1.0	272
G25♀																
1/25/40	4.90	11.17	41.0	83	23	27	1.2	7.0	2	1	45	47	1.0	—	4.0	236
G28♀																
1/23/40	4.79	10.14	35.2	74	21	29	0.8	14.9	—	—	43	53	2.0	1.0	1.0	168
G29♀																
1/23/40	4.89	10.70	36.7	75	22	29	0.6	9.0	1	1	68	27	—	—	3.0	270
G33♀																
1/22/40	4.20	10.35	35.4	84	25	29	2.4	10.7	—	—	61	34	2.0	1.0	2.0	240
G35♀																
1/22/40	4.70	11.17	39.0	84	24	29	1.0	9.0	—	—	49	30	5.0	1.0	15.0	110
G38♀																
1/22/40	4.30	10.35	33.0	77	24	31	3.2	14.3	—	—	57	28	3.0	—	12.0	260

[1] Mean values of observations made by Shukers, Langston and Day in normal animals.

[2] Mean values of our observations in normal animals.

and 3 subcutaneous injections of 30,000 I.U. of estradiol propionate weekly until June 30, when injections were discontinued for the summer. Three 3-mg. pellets of estradiol dipropionate were implanted 8 times between Oct. 23, 1939 and Jan. 23, 1940, when a blood specimen was taken (table 1).

No. G 29 (wt. 2480 gm.) also oöphorectomized, treated in the same manner as *G 28*.

No. G 33 (wt. 3360 gm.). Oöphorectomized. From Oct. 23, 1939 until Jan. 22, 1940, when the blood sample was taken, this female received 30,000 rat units of estradiol propionate in oil (1 cc. containing 30,000 R.U.) 3 times weekly, and five 3-mg. pellets of the same compound every 2 weeks until the blood sample listed in table 1 was taken, a total of 112,000 R.U. in oil plus the amount absorbed from the pellets.

No. G 35 (wt. 3250 gm.) and *No. G 38* (wt. 3100 gm.) had been oöphorectomized. Same treatment as animal *G 33*.

Significant changes, as for instance the granulocytopenia seen in dogs, were not encountered in our animals. *Monkeys 604* and *606* showed during the treatment a certain decline of the red blood count, hemoglobin and volume of packed red blood cells. The lowest red cell count in these two animals, on March 14, was accompanied by a higher mean corpuscular volume, though without any increase in the amount of hemoglobin per cell. Following the injection of progesterone (plus testosterone propionate in *monkey 606*) these values showed a tendency to change in the opposite direction. The other animals did not reveal any significant changes. The white blood cells showed a striking individual variation, which appears also in the data of Shukers, Langston and Day (3) and in the work of others.

SUMMARY

A study of the blood picture in the rhesus monkey revealed no toxic effect of large doses of estrogens, a result quite different from that seen in the dog.

We wish to thank Dr. M. M. Wintrobe for his helpful advice.

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EFFECT OF ACUTE ESTRADIOL OVERDOSAGE ON THE UTERUS OF THE GUINEA PIG[1]

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THE MARKED EFFECTS of follicular extracts on the uterus of the guinea pig were first observed by Loeb and Kountz (1); they noted "swelling, hyperemia, slight localised edema, and some hemorrhage in the mucosa." Césa (2) later reported an active growth of uterine epithelium with marked edema after treating guinea pigs with follicular extracts. This increase in water content of the uterus has recently been used as a quantitative test (3) for the determination of estrogens. Nelson (4, 5) in a number of reports, published results on a large series of guinea pigs treated with

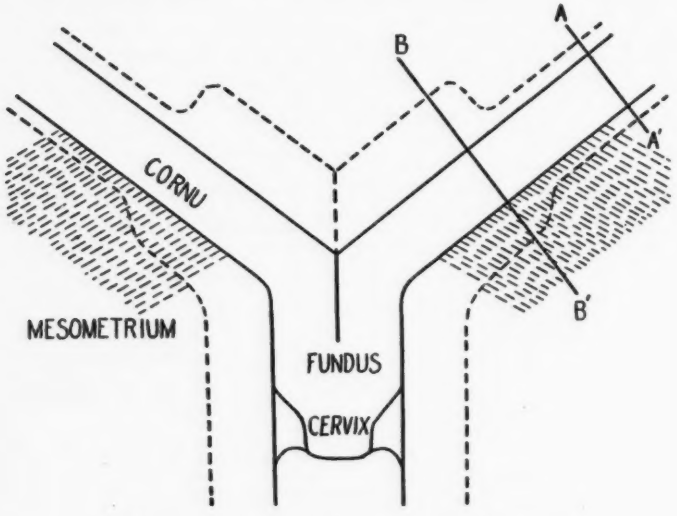

Fig. 1. DIAGRAM OF NORMAL GUINEA PIG UTERUS (SOLID LINE), α-ESTRADIOL TREATED (BROKEN LINE).

weekly doses of 60 to 100γ of estradiol for 6 months; there was a constant occurrence of cystic glandular hyperplasia starting in the cornua and finally extending to the fundus, with metaplasia of the epithelial cells especially in the cervical region. At about the same time Dessau (6, 7) reported on the effects of daily doses of 7γ of estradiol for 3 months on the guinea pig uterus. He found hyperplasia in both endometrium

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[1] A brief abstract of this work was presented before the American Association of Anatomists at its 57th Meeting in Chicago, April 9–11, 1941.

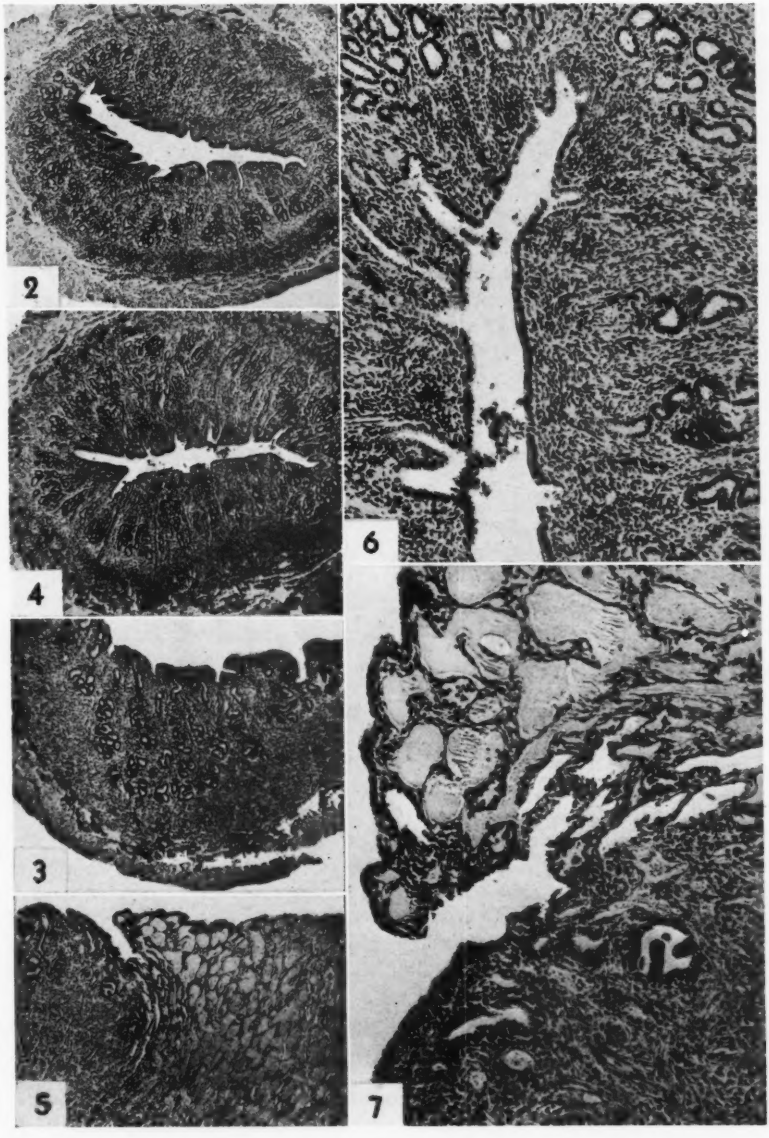

Fig. 2. Low power microphotograph of cross section of normal guinea pig uterus through level AA[1] (fig. 1). Fig. 3. Low power microphotograph of cross section of estrogen treated guinea pig at same level as figure 2. Note the enlargement partly due to glandular hypertrophy, but mainly to distention of the lumen. Fig. 4. Low power microphotograph of cross section of normal guinea pig uterus through level BB[1] (fig. 1). Fig. 5. Low power microphotograph of cross section of estrogen treated guinea pig uterus at same level as figure 4. Note relatively sharp demarcation line between comparatively normal zone to the left and extensive hyperemia to the right of the field. Fig. 6. High power microphotograph corresponding to figure 4. Fig. 7. High power microphotograph corresponding to figure 5 showing detail. Loss of epithelium and excessive vascular dilatation are evident in upper part of section.

and myometrium accompanied by striking changes in the epithelium which started in the fundus and proceeded to the cornua—changes from the normal simple columnar to a mucous secreting type and finally to stratified squamous.

In order to study the effect of very large doses of an estrogenic hormone over a short period of time on the uterus of the guinea pig, we performed an experiment on 12 adult females averaging 300 gm. in body weight. Six of those received daily subcutaneous injections of 5 mg. of α-estradiol dissolved in 0.5 cc. of peanut oil for a period of 20 days, while the remaining 6 animals served as controls and were treated with 20 mg. of cholesterol which was administered in the same manner. On the 21st day the guinea pigs were killed, and the organs fixed, weighed and examined histologically. The ovaries, as expected, had doubled their size in the estrogen treated group, averaging 49 mg. as compared to 22 mg. in the control group; this increase in size was mainly due to intense stimulation of follicle growth.

The changes in the size of the uteri were even more marked. In the cholesterol group they averaged 434 mg. (range: 312–618) as against an average of 1.253 gm. (range: 0.966–2.092) in the estradiol group. The body of the uterus in all of the latter group of animals was greatly and uniformly enlarged but even gross inspection showed a distinct difference in the response of the horns. Although in the control animals the cornua of the uteri were of approximately the same appearance throughout their length, in those treated with α-estradiol the caudal third of the horns was greatly enlarged and dark red in color, similar to that seen in the fundus. The cranial two-thirds were only slightly enlarged and comparatively pale in color. In order to study this striking difference further, we made sections of the cranial (level AA^1, fig. 1) and caudal parts (level BB^1) in all cases.

A low power magnification through the cranial part of the cornua of a control animal shows the endometrial glands and simple columnar epithelium which are characteristic of the normal uterus (fig. 2).

Sections at the same magnification of the corresponding area in the estrogen treated guinea pig show only some enlargement of the endometrial glands and stroma, but this increase in size is mainly due to distension of the lumen with fluid (probably secreted at a lower level). The epithelium is normal and the blood vessels have not been affected (fig. 3.).

Sections made across the caudal part of the cornua of a control guinea pig are practically identical with figure 2 (fig. 4, 5). In the corresponding area of an estrogen treated animal, there has been intense dilatation and a striking change in the whole appearance of the mucosa which actually gives the impression of a cavernous body (fig. 6). Very extensive hyperemia has occurred in the endometrial stroma accompanied by necrosis of the glands and degeneration of the surface epithelium with sloughing into the lumen.

If we examine a cross section of the cornua in this area under a lower magnification (fig. 7), we observe that this change is much more advanced in one quadrant of the section and that a distinct demarcation line between the two zones is quite obvious. It is of interest to note that the area of greatest change in all cases is on the antimesometrial side.

In the fundus we find that the area close to the septum is very similar in appearance to the (beginning) caudal end of the cornua, with loss of epithelium and degeneration of the glands. But towards the cervix these changes become less marked although the intense hypertrophy persists.

SUMMARY AND CONCLUSIONS

In summary we may say that although acute overdosage with α-estradiol produces marked increase in the size of the whole uterus, the parts of this organ, although normally of the same histological appearance throughout, do not respond uniformly to treatment. The area of greatest response seems to be along the cranial part of the fundus and the caudal third of the cornua, especially on the antimesometrial side, and the sensitivity to the hormone decreases both towards the cranial end of the cornua and towards the cervix.

We may conclude that this differential responsiveness of the various parts of an organ, which normally exhibits a fairly uniform structure indicates that organs or parts of organs having the same histological appearance may differ in their hormone sensitivity.

The expenses of this investigation were defrayed by the Blanche E. Hutchinson Research Fund of McGill University. The author is greatly indebted to Drs. Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey, for the estradiol used in these experiments.

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COMPARATIVE ESTROGENIC POTENCY OF DIETHYL STILBESTROL, ESTRONE, ESTRADIOL AND ESTRIOL

II. UTERINE AND VAGINAL CHANGES IN INFANTILE RATS

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SINCE the initial report of Dodds, Goldberg, Lawson and Robinson (1) on the preparation and estrogenic properties of stilbestrol (diethyl stilbestrol), numerous studies have been made on the physiological properties of this compound.

We have previously reported (2) the comparative oral and subcutaneous potency of stilbestrol, estrone, α estradiol and estriol, on spayed adult rats and mice.

As an extension of these studies we have compared the effects of oral and subcutaneous administration of these estrogens on the uterine weight and vaginal, uterine and ovarian histology of infantile rats.

TABLE 1. THE EFFECT OF SUBCUTANEOUS ESTROGENS ON THE UTERINE WEIGHT AND VAGINAL ESTRUS OF IMMATURE FEMALE RATS

No. rats	Total dose	Mean uterine wt.	Stand. error E[1]	Estrus	No. rats	Total dose	Mean uterine wt.	Stand. error E[1]	Estrus
	γ	mg.		%		γ	mg.		%
Stilbestrol					*Estrone*				
24	control	15.3	0.4	0	24	control	15.3	0.4	0
6	0.01	16.8	0.6	0	5	0.20	15.1	0.6	0
5	0.015	18.4	0.9	0	10	0.25	17.0	0.3	0
11	0.02	21.5	0.7	0	10	0.35	20.0	0.9	30
6	0.025	28.2	0.6	50	5	0.40	27.3	2.3	60
10	0.05	31.5	0.9	100	13	0.50	28.9	1.4	50
13	0.08	52.0	1.2	100	9	1.00	30.5	1.5	90
4	0.40	55.0	3.1	100	6	2.50	39.5	3.6	70
7	1.00	56.5	4.1	100	6	5.00	39.9	2.3	100
4	2.00	66.0	1.5	100	5	7.50	46.0	1.7	100
2	6.00	65.0		100	3	10.00	46.4	1.8	100
α Estradiol					*Estriol*				
24	control	15.3	0.4	0	24	control	15.3	0.4	0
7	0.03	15.6	0.6	0	5	0.015	15.0	0.5	0
13	0.05	20.3	0.5	0	5	0.025	16.5	0.8	0
5	0.10	24.6	1.6	40	12	0.05	18.7	0.5	60
9	0.15	31.3	1.0	90	8	0.10	17.5	0.8	50
7	0.25	33.5	1.0	100	5	0.15	19.4	0.8	60
7	0.50	35.3	1.1	100	9	0.50	21.2	0.6	100
7	1.00	37.2	1.8	100	8	1.00	22.5	0.6	100
7	2.00	40.1	1.2	100	4	1.50	22.2	2.2	100
2	9.00	39.0		100	3	15.00	20.7	1.0	100

[1] $E = \sqrt{\frac{\Sigma d^2/n-1}{n}}$.

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Bülbring and Burn (3) found that assays utilizing the uterine weight of spayed infantile rats were as accurate, more rapid and more economical than assays using the vaginal smear method. In a recent study Andersen and Pedersen-Bjergaard (4) indicate that assays of estrone on infantile spayed female rats, using the uterine weight method yield results more accurate than when the Allen-Doisy vaginal smear method is used. Other investigators, notably Dorfman, Gallagher and Koch (5); Lauson, Heller, Golden and Sevringhaus (6) and Dorfman (7) have studied the comparative subcutaneous effect of naturally occurring estrogens upon the uterine weight of infantile rats.

TABLE 2. THE EFFECT OF ORAL ESTROGENS ON THE UTERINE WEIGHT AND VAGINAL ESTRUS OF IMMATURE FEMALE RATS

No. rats	Total dose	Mean uterine wt.	Stand. error E[1]	Estrus	No. rats	Total dose	Mean uterine wt.	Stand. error E[1]	Estrus
	γ	mg.		%		γ	mg.		%
Stilbestrol					*Estrone*				
24	control	15.3	0.4	0	24	control	15.3	0.4	0
7	0.03	15.1	0.9	0	5	0.5	16.6	0.8	40
5	0.03	18.0	1.3	0	7	1.0	19.1	0.7	40
10	0.06	19.2	0.7	0	5	2.0	23.3	1.2	60
14	0.10	20.7	0.6	35	8	3.0	28.8	0.5	75
11	0.15	28.6	1.0	100	6	4.0	38.8	1.3	100
6	0.20	30.9	1.2	85	7	8.0	44.5	1.5	100
9	0.30	41.7	2.0	100	8	12.0	46.5	2.5	100
6	1.50	48.2	1.1	100	5	100.0	47.4	1.5	100
5	3.00	49.7	1.2	100					
α Estradiol					*Estriol*				
24	control	15.3	0.4	0	24	control	15.3	0.4	0
5	0.25	16.0	1.3	20	5	0.01	14.8	0.9	0
8	0.5	20.3	1.7	65	6	0.05	20.6	0.7	0
7	0.8	21.6	1.2	60	8	0.10	20.9	0.7	0
7	1.0	23.2	0.6	45	5	0.25	21.0	0.7	20
7	2.0	32.8	3.6	100	8	0.50	18.5	1.1	80
6	4.0	40.4	2.9	100	6	1.00	23.5	0.8	70
6	6.0	47.0	3.5	100	5	2.00	25.7	1.4	100
4	25.0	46.5	1.3	100	6	3.00	26.5	0.9	85
5	50.0	48.1	0.9	100	6	6.00	31.2	1.0	100
					4	12.00	29.1	0.4	100
					3	24.00	28.4	1.6	100

[1] $E\sqrt{\frac{\Sigma d^2/n-1}{n}}$.

Recently Von Haam, Hammel, Rardin and Schoene (8) studied the uterine weight changes in infantile mice produced by stilbestrol and estrone.

EXPERIMENTAL

Female Wistar strain albino rats 21 to 23 days old, weighing 37 to 41 gm. were selected for the tests. Subcutaneous injections of the estrogens were made in 0.05 cc. vegetable oil once daily for 3 days; oral injections were made twice daily in 0.25 cc. water, utilizing a stomach tube method described by Tandy and Pugh (9). The animals were killed 96 to 100 hours after the initial injection. Vaginal smears were made on the animals that had open vaginae (or easily opened) and the animal autopsied. Ovaries, uteri and vaginae were dissected free from surrounding tissue and fixed 24 to 48 hours in Bouin's fixative. The uteri were transferred to 70 to 80%

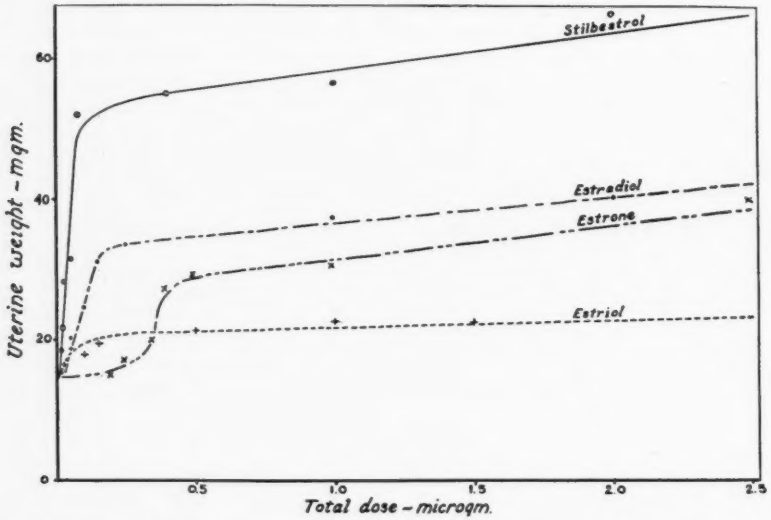

Fig. 1. Effect of graded subcutaneous doses of estrogens on the uterine weight of immature Rats.

alcohol and then carefully blotted between sheets of toweling to express the intrauterine fluid, and weighed on an analytical balance. Representative samples of ovaries, uterus and vagina were prepared for histological study.

RESULTS

Gross experimental observations of the effects of subcutaneous estrogens are recorded in table 1. The total dose of stilbestrol, estrone, α-estradiol and estriol which produced the following changes were: minimum significant uterine weight change

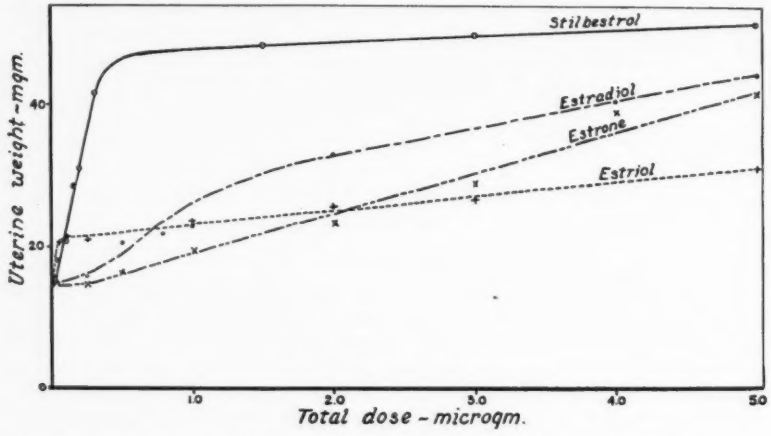

Fig. 2. Effect of graded oral doses of estrogens on the uterine weight of immature rats.

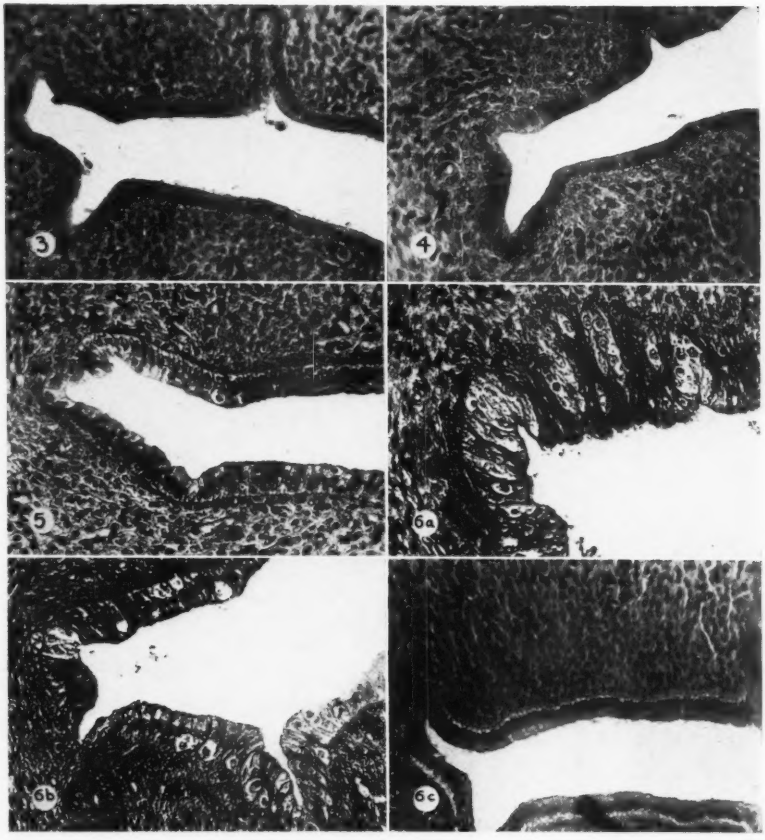

Fig. 3. Uterus, Stage 0. Fig. 4. Uterus, Stage 1. Fig. 5. Uterus, Stage 2. Fig. 6a. Uterus Stage 3 (maximum stimulation from stilbestrol). Fig. 6b. Maximum uterine stimulation from estrone or estradiol. Fig. 6c. Maximum uterine stimulation from estriol.

0.015γ, 0.2γ, 0.05γ and 0.05γ respectively; initial vaginal estrus 0.025γ, 0.35γ, 0.10γ and 0.05γ respectively; maximum average uterine weight 2.0γ (65 mg.), 7.0γ (46 mg.), 2.0γ (40 mg.) and 1.0γ (22.5 mg.) respectively.

Gross experimental observations of the effects of oral estrogens are recorded in table 2. The total dose of stilbestrol, estrone, α-estradiol and estriol which produced the following changes were: minimum significant uterine weight change 0.03γ, 1.0γ, 0.5γ and 0.05γ respectively; initial vaginal estrus 0.10γ, 0.5γ, 0.25γ and 0.25γ respectively; maximum average uterine weight 1.5 (49 mg.), 8.0γ (45 mg.) 6.0γ (46 mg.) and 6.0γ (31 mg.) respectively.

Figures 1 and 2 present the effects of graded doses of estrogens administered subcutaneously and orally. Table 3, based on experimental evidence, lists the effects one would expect from doses of estrogens and shows the relative potency of the various compounds when compared with stilbestrol.

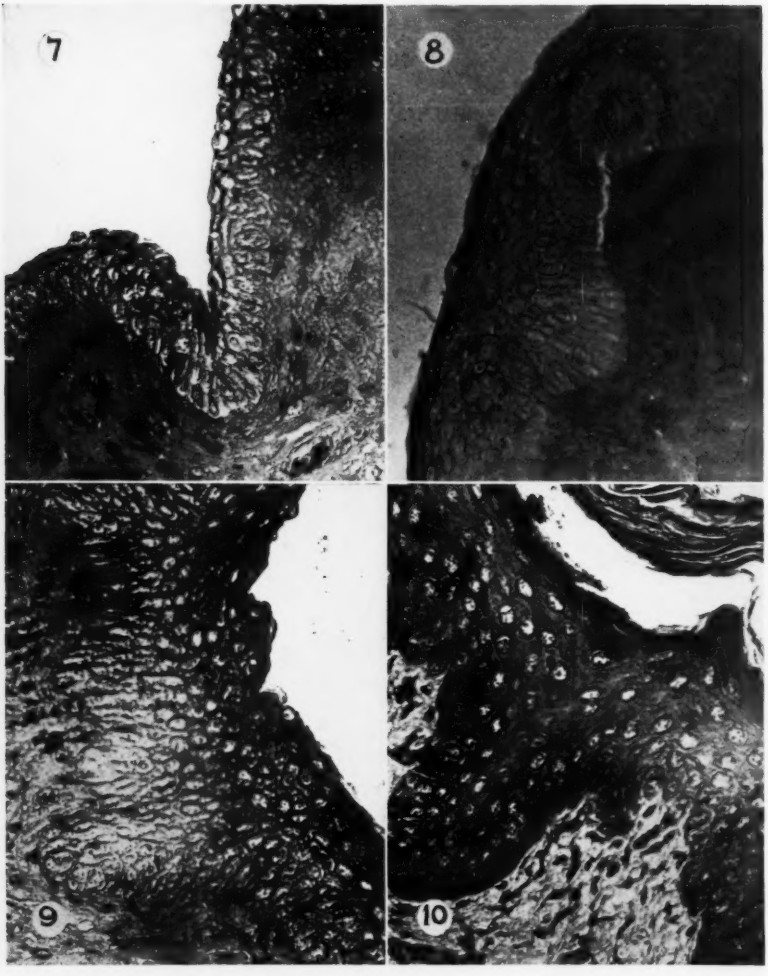

Fig. 7. VAGINA, STAGE 0. Fig. 8. VAGINA, STAGE 1. Fig. 9. VAGINA, STAGE 2. Fig. 10. VAGINA, STAGE 3.

Histological Study

The ovaries were apparently unchanged by the dosage of estrogens administered. As would be expected the most striking changes were confined to the endometrium and vaginal mucosa. For the most part the histological changes were reflected in the gross uterine weight (table 4) and vaginal smear changes.

The histological changes in the endometrium have been arbitrarily divided into 4 stages. *Stage* 0 (fig. 3) is normal relative to uninjected controls and consists of an epithelium of low columnar cells containing little cytoplasm and having a vertical diameter of 2 to 3 times the transverse diameter. The stroma cells were fairly closely packed and contained little cytoplasm. *Stage* 1 (fig. 4) exhibits a somewhat taller

TABLE 3. THE COMPARATIVE EFFECT OF GRADED DOSES OF SUBCUTANEOUS AND ORAL ESTROGENS ON THE IMMATURE RAT UTERUS

Uterine wt. change		Subcutaneous dose							Oral dose						
		Stil-bestrol	Estrone		Estradiol		Estriol		Stil-bestrol	Estrone		Estradiol		Estriol	
Wt., mg.	%	γ	γ	ratio to stilb.	γ	ratio to stilb.	γ	ratio to stilb.	γ	γ	ratio to stilb.	γ	ratio to stilb.	γ	ratio to stilb.
15	Nil	0.01	0.20	20	0.03	3.0	0.015	1.5	0.02	0.4	20	0.1	5	0.01	0.5
18	20	0.015	0.30	20	0.04	2.7	0.06	4.0	0.05	1.0	20	0.3	6	0.03	0.6
20	30	0.02	0.35	18	0.05	2.5	0.03	15.0	0.075	1.5	20	0.5	7	0.05	0.7
23	50	0.023	0.38	17	0.08	3.5	1.00	40.0	0.125	2.0	16	1.0	8	1.00	8.0
25	65	0.025	0.40	16	0.10	4.0	15.00		0.15	2.3	15	1.3	9	2.00	13.0
30	100	0.04	0.60	15	0.15	3.8			0.20	3.1	15	1.7	10	6.00	30.0
35	130	0.05	1.50	30	0.50	10.0			0.25	3.6	14	2.5	12	24.00	
40	165	0.06	2.50	42	2.00	33.0			0.30	4.2	14	4.0	13		
45	200	0.07	7.50	107	9.00				0.50	7.0	14	7.0	14		
50	230	0.08	10.00						3.00	100.0		50.0			
65	330	2.00							15.00						
min. dose producing 50% estrus		0.025	0.4	16	0.10	4	0.05	2	0.125	1.0	8	0.5	4	0.4	3

epithelium, the cells having a vertical diameter of 3 to 4 times the transverse, the principal increase being due to supranuclear cytoplasm. The stroma is still essentially dense in nature. *Stage 2* (fig. 5) consists of a tall epithelium, approximately one-half of the cell being composed of granular supranuclear cytoplasm. The vertical diameter is about 5 times the transverse diameter. The stroma cells are somewhat hyperplastic and have considerable cytoplasm. *Stage 3* (fig. 6a, 6b) consists of a tall columnar epithelium containing numerous vacuolated cells, the vertical diameter being 5 to 10 times the transverse. The glands seem to be more numerous and deeper. The stroma is hyperplastic, the cells containing considerable cytoplasm and their sections being approximately twice the normal area.

TABLE 4. UTERINE AND VAGINAL HISTOLOGY OF IMMATURE RATS RECEIVING ESTROGENS AT VARIOUS DOSE LEVELS

Histological Stage[1]	Estrogenic material	Uterus		Vagina	
		Subcutaneous dose	Oral dose	Subcutaneous dose	Oral dose
		γ	γ	γ	γ
Zero figures 1 & 5	Stilbestrol	0.0 – 0.05	0.0 – 0.3	0.0 – 0.01	0.0 – 0.1
	Estrone	0.0 – 0.5	0.0 – 2.5	0.0 – 0.35	0.0 – 0.5
	α estradiol	0.0 – 0.12	0.0 – 1.5	0.0 – 0.05	0.0 – 0.25
	Estriol	0.0 – 0.3	0.0 – 2.0	0.0 – 0.15	0.0 – 0.10
One figures 2 & 6	Stilbestrol	0.025– 0.05	0.10– 0.35	0.01 – 0.025	0.1 – 0.2
	Estrone	0.35 – 1.0	1.0 – 3.5	0.35 – 0.5	0.5 – 2.0
	α estradiol	0.05 – 0.2	0.5 – 2.5	0.03 – 0.15	0.25– 1.0
	Estriol	0.3 –15.0	0.05– 5.0	0.1 – 1.0	0.01– 2.0
Two figures 3 & 7	Stilbestrol	0.04 – 0.1	0.2 – 3.0	0.025– 0.1	0.1 – 0.3
	Estrone	0.6 – 2.5	3.0 – 7.0	0.3 – 2.5	1.0 – 6.0
	α estradiol	0.15 – 2.0	1.5 – 7.0	0.1 – 1.0	0.5 – 6.0
	Estriol		5.0 – 12.0	0.05 –15.0	0.4 – 6.0
Three figures 4, 4a, & 8	Stilbestrol	0.08 – 6.0	0.5 – 15.0	0.06 – 6.0	0.3 – 15.0
	Estrone	2.5 –10.0	4.0 –100	1.0 –10.0	3.0 –100
	α estradiol	2.0 – 9.0	4.0 – 50	0.5 – 9.0	3.0 – 60
	Estriol			0.5 –15.0	5.0 – 25

[1] See histological descriptions.

Figures 6a, 6b and 6c show the contrasts of the maximum degree of stimulation observed in the endometrium of rats receiving stilbestrol, estrone or α-estradiol, and estriol respectively.

The vaginal responses have also been divided into 4 stages. *Stage 0* (fig. 7) is composed of non-differentiated epithelium with a poorly differentiated basement structure; this is normal for the controls. *Stage 1* (fig. 8) shows an early differentiation of the superficial squamous structure and a denser basement structure. *Stage 2* (fig. 9) shows a well differentiated estrous mucosa, with several superficial layers of stratified squamous epithelium and a well formed basement structure. *Stage 3* (fig. 10) illustrates a completely estrous vaginal mucosa with cornified cells at the surface. Note the irregular peg-like basement structure.

DISCUSSION

It is apparent, under the experimental conditions of this study, that: the degree of uterine stimulation obtained from stilbestrol exceeds that obtainable from any of the natural estrogens; (this fact may differentiate it from natural estrogens) the oral doses of stilbestrol have approximately the same effect as equivalent subcutaneous doses of estradiol; orally, estriol is approximately equivalent to stilbestrol in producing initial estrous changes in the vagina and uterus, but as has been generally observed by others these changes reach an early maximum (Dorfman, et al. made use of this fact to biologically differentiate estriol from estrone).

SUMMARY

The comparative estrogenic activity of stilbestrol, estrone, α-estradiol and estriol by oral and subcutaneous administration to infantile female rats has been presented.

The comparative activity of estrone, α-estradiol and estriol was of the same order as observed by Dorfman, Gallagher and Koch and Lauson, Heller, Golden and Sevringhaus.

Assayed subcutaneously estrone, α-estradiol and estriol were found to be respectively, $\frac{1}{18}$, $\frac{1}{4}$, and $\frac{1}{10}$ as effective as stilbestrol; orally the relationship was $\frac{1}{16}$, $\frac{1}{8}$ and $\frac{1}{3}$.

Stilbestrol orally had about the same activity as an equal subcutaneous dose of α-estradiol.

The degree of stimulation from stilbestrol observed grossly or histologically, exceeded that attained by any of the natural estrogens.

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COMPARATIVE STUDIES OF THE EFFECTS OF ESTRADIOL AND STILBESTROL UPON THE BLOOD, LIVER, AND BONE MARROW[1]

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THE SYNTHETIC ESTROGEN, 4,4-dihydroxy, $\alpha\beta$ diethylstilbene (stilbestrol) has been subjected to intensive clinical investigation during the past 2 years. It has been demonstrated that this diphenolic estrogenic substance is capable of relieving the symptoms brought about by a deficiency of the ovarian hormone and that it has a high potency when it is compared with the available estrogens derived from natural sources. In some instances, however, the administration of stilbestrol has been accompanied by unpleasant symptoms, the most frequent of which is nausea, either with or without vomiting. The reported incidence of nausea as reported by different investigators has ranged from 7 to 80%. Among other less frequent disagreeable manifestations which have been reported are abdominal cramps, headaches, edema, rashes, jaundice, salivation, diarrhea and paresthesia. It has been suggested, but without conclusive evidence, that the liver may be damaged. The suspicion of hepatic damage has arisen because of both nausea and vomiting and of instances of jaundice following the administration of stilbestrol.

Efforts to demonstrate hepatic injury by functional tests have yielded conflicting results. Shorr, Robinson and Papanicolau (1) reported their experiences with stilbestrol in a series of 44 women. They observed an unusually high incidence of side actions and their studies of hepatic function yielded equivocal results. They concluded that the use of stilbestrol should be confined to experimental studies in the human being by qualified investigators. In the 72 patients to whom we have administered stilbestrol, no evidence of hepatic disturbance was encountered by the tests we employed (2). Nausea with or without vomiting occurred in only 15% of the patients. Nausea occurred after parenteral as well as after oral administration of the drug, and therefore cannot be considered the result of direct gastrointestinal irritation. The tendency to nausea was overcome in all but 4 instances by reducing the dose or by changing from oral to parenteral administration. Whether the nausea and vomiting are signs of more serious organic disturbance or whether they may be only functional disturbances has yet to be determined. Recently (3) large doses of stilbestrol were administered to 3 patients suffering with terminal carcinoma. Daily doses of 10 to 25 mg. were given over a period of 10 to 45 days, the total amount ranging from 250 to 1125 mg. Post mortem examination of these patients revealed no evidence of hepatic damage other than the changes often observed in those who die after a prolonged illness. Freed, Rosenbaum and Soskin (4), and von Haam and co-workers (5) studied the effects of stilbestrol administration on menopausal women and found no evidence of hepatic impairment. The hepatic changes noted in experimental animals following the administration of stilbestrol

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[1] A preliminary report of these studies was presented before the American Society for Clinical Investigation, Atlantic City, May 6, 1940.

have been described as fatty degeneration (6, 7) and interstitial hepatitis (8). The fatty degeneration of the liver was observed in both dogs and rats and the interstitial hepatitis was noted only in rats. In considering the toxic manifestations of stilbestrol it must be emphasized that large doses of estrogens derived from natural sources may also produce fatty degeneration of the liver and interstitial hepatitis in dogs (9, 10).

Although only slight or questionable hepatic changes have been produced with stilbestrol in experimental animals, striking effects are demonstrable in the bone marrow and in the peripheral blood. There is conclusive evidence that changes in the bone marrow and peripheral blood can be produced by the administration of either natural (10, 11, 12, 13, 14) or synthetic (7, 13, 15) estrogens.

In considering the toxicity of both stilbestrol and the natural estrogens it is well to note that there seems to be a marked species suceptibility. In dogs, estrogens given in large doses readily produce extensive alteration of the bone marrow and the formed elements of the peripheral blood. When the rat is chosen as the experimental animal, a relative resistance to the estrogens is observed, although anemia and leucocytosis may be produced when enormous quantities of the drugs are used. However, there is a tendency to recovery. Tyslowitz (16), by studying changes in the peripheral blood, reports that the rhesus monkey is less susceptible than the dog to the toxic effects of large doses of estradiol.

To date no evidence of toxic effects on the blood and bone marrow following the clinical use of stilbestrol has been advanced. The report (17) of intermittent purpura hemorrhagica associated with menstruation and periodic fluctuation of the thrombocyte count may be related to estrogenic effects. In normal women there is a variation in the total number of platelets as related to the menstrual cycle (18–20).

MATERIALS AND METHODS

Studies were made on 17 male and female dogs, 5 of which were used for controls. All were kept for a conditioning period of 7 to 14 days in the animal quarters before the actual experiments were undertaken. A diet of Purina Dog Chow was used. During the control period, red, white, differential and platelet counts were done on the blood. Only those animals with a normal blood picture were selected for use. The following determinations were made at intervals throughout the experiment: icteric index, bromsulphthalein hepatic function test, prothrombin time and blood calcium estimation. Red and white cell counts, differential and platelet counts were determined on the blood at 2-day intervals on the animals receiving the hormone by injection, and at weekly intervals on those receiving the drug by mouth. When there was evidence of a developing thrombocytopenia, the blood counts were taken daily until death occurred. The animals given the smaller doses of estrogenic material survived for long periods and were killed when the studies were completed. Autopsies were performed routinely. Although all tissues were preserved, only the findings in the liver and bone marrow are being reported at this time.

In order that comparative observations could be made between stilbestrol and estradiol, the estrogenic potency was calculated in terms of international units. One milligram of diethylstilbestrol dipropionate is equivalent to 20,000 international units (I.U.). One milligram of alpha estradiol is equivalent to 12,000 rat units (R.U.), while estradiol benzoate has a potency of 6,000 R.U. per mg. Although there is some difficulty in comparing the R.U. of estradiol with the I.U. of estrone, it is widely accepted that 1 R.U. is roughly equivalent to 10 I.U. When given by injection 5 mg. of stilbestrol dipropionate may be considered approximately equivalent to 1.66 mg. of estradiol benzoate (10,000 R.U. or 100,000 I.U.).

Crystalline diethylstilbestrol dipropionate was weighed into gelatin capsules containing 1 mg., 10 mg., or 100 mg. for oral administration. Diethylstilbestrol was used in the form of 5 mg. tablets. For parenteral use, diethylstilbestrol dipropionate in sesame oil was used in doses of 1 mg., 5 mg., 10 mg., or 100 mg. Estradiol benzoate in

sesame oil was injected intramuscularly in doses of 1.66 mg. and 3.32 mg. Alpha estradiol was fed to one animal mixed in ground meat in a daily dose of 16.25 mg.[2]

OBSERVATIONS

Five control dogs were maintained on the same diet and under the same general conditions as all of the animals used in experiment. These animals maintained their weight and showed no great variation in the peripheral blood elements. The normal range of the formed blood elements were: erythrocytes, 5,500,000 to 7,500,000; leucocytes, 10,000 to 20,000; platelets, 900,000 to 1,500,000; hemoglobin, 11 to 15 gm.

Male and female dogs injected daily with large doses of estrogens developed thrombocytopenia, manifested by gingival bleeding and petechiae of the conjunctiva and mucous membranes. Subsequently there developed either acute or subacute gastro-intestinal, pulmonary or subcutaneous hemorrhages. In the terminal stages the animals were anorexic and cachectic. Dogs receiving the smaller doses of estrogens appeared and behaved normally throughout the course of the experiments (table 1).

Changes in the blood counts. Qualitatively the changes in the blood following stilbestrol treatment were indistinguishable from those which were observed following estradiol. Quantitatively, when given in equivalent doses, estradiol benzoate produced more rapid and more severe changes in the blood. In each group the first alteration consisted of a rise in the number of leucocytes with an almost simultaneous fall of the thrombocytes. In some instances there was a disappearance of platelets from the circulating blood. In some animals the leucocyte count reached a high level, in one case 133,000. If the animal survived a sufficient length of time, the leucocytosis regressed until a marked leucopenia occurred. With the increase in the number of granulocytes there was a moderate decrease in the red cells with a proportionate drop in the hemoglobin. This change was observed before any evidence of hemorrhage appeared. In those animals which developed hemorrhage while the leucocyte count was at a peak, a reticulocyte response was not observed. Those animals dying with pronounced leucocytosis had a hyperplasia of the myeloid elements of the bone marrow with a decrease in the foci of erythropoiesis. Comparative curves showing the changes in the various blood elements are shown in figures 1 and 2.

Animals receiving the smaller doses of estradiol and stilbestrol developed mild to moderate anemia from which they tended to recover even while the same doses of the estrogens were continued (table 1).

Changes in the bone marrow. Studies of the bone marrow revealed definite abnormalities. When doses of equivalent estrogenic potency were given, these effects appeared more rapidly with estradiol benzoate than with stilbestrol. The first characteristic change consisted of a variable degree of hyperplasia, usually of the myeloid elements. As this progressed, scattered areas of focal hypoplasia were noted. These foci usually consisted of a tenuous, edematous stroma, sometimes containing a few erythrogenic cells, and less frequently, immature myeloid cells. In one case essentially the entire bone marrow became hypoplastic (3,700 mg. diethylstilbestrol dipropionate by mouth in 37 days). Frequently the megakaryocytes were reduced in numbers and those that remained were shrunken, with deeply staining basophilic nuclei (table 1).

Changes in the liver. The livers of the animals did not reveal constant pathological changes either after estradiol or after stilbestrol administration. Of the 6 animals receiving stilbestrol by mouth, 4 showed some fatty degeneration, 1 moderate central necrosis, and 2 showed no hepatic change. Of the 3 dogs receiving estradiol, 2 showed

[2] Stilbestrol was supplied for these studies by the Department of Medical Research, Winthrop Chemical Company, New York City. Estradiol was supplied by the Medical Research Division of the Schering Corporation, Bloomfield, New Jersey.

TABLE I

Dog No.	Drug	Daily dose, mg.	Days survived	Day of count	Blood studies						Bone marrow changes	Hepatic changes
					WBC	R.B.C.	Hgb gm.	Hgb %	Retics	Platelets		
Oral Administration												
1	Stilb. diprop.	100	37	1	14,900	5.58	11.2	72	0.8	703,000	Marked hypoplasia; granulopoiesis minimal; erythrocytic elements greatly reduced	Slight fatty degeneration liver cells in central zone
				20	72,400	4.45	11.1	72	1.0	89,000		
				26	119,500	2.39	6.0	39	0.6	4,700		
				37	2,150	1.30	2.2	14	8.6	6,000		
13	Stilb.	10	43[1]	1	13,800	4.99	12.7	83	1.4	1,057,000	Increase in marrow cellularity due to increase of myeloid element, megakaryocytes reduced in number	Minimal fatty degeneration; one focus of necrosis
				30	43,000	5.14	11.7	76	1.0	781,000		
				37	27,000	4.49	11.8	77	1.0	898,000		
7	Stilb.	10	20[2]	1	15,500	5.07	9.7	63	1.6	963,000	Cellularity increased, immature myeloid elements predominate	Occasional focus of necrosis; moderate fat in portal zones
				15	29,000	5.08	9.0	59	0.8	944,000		
6	Stilb. diprop.	1	128	1	32,000	7.42	15.7	101	1.4	1,850,000	Mild increase in cellularity, immature myeloid cells predominate	Minute amount of fat in liver cells
				26	36,000	6.16	13.8	90	2.4	1,600,000		
				78	14,250	4.86	10.0	66	1.2	544,000		
				128	18,000	6.20	13.0	84	3.6	1,470,000		
3	Stilb. diprop.	10	186	1	9,900	5.82	14.7	95	1.4	954,000	Essentially normal	Essentially normal
				22	56,000	5.22	10.5	68	0	995,000		
				80	22,000	4.33	10.1	66	10.2	757,000		
				180	9,050	5.39	13.5	87	3.6	1,720,000		
11	α estradiol	16.25	135	1	11,900	6.47	15.9	103	1.0	1,560,000	Foci showing decrease in polymorphonuclears; megakaryocytes show minimal decrease	Esentially normal
				22	26,900	5.00	12.0	78	0	225,000		
				35	26,000	4.69	10.0	65	4.6	281,000		
				85	10,900	5.33	12.7	83	2.0	1,080,000		
				135	13,000	5.00	13.2	85	1.0	830,000		

TABLE 1—*Continued*

Dog No.	Drug	Daily dose mg.	Days survived	Day of count	Blood studies: WBC	R.B.C.	Hgb gm.	Hgb %	Retics	Platelets	Bone marrow changes	Hepatic changes
					Injection							
2	Stilb. diprop.	10	21	1	15,600	7.30	14.4	98	0	1,500,000	Slight hyperplasia of myeloid series; erythropoiesis unaltered	Moderate central necrosis; central fatty degeneration
				14	28,000	7.00	12.9	84	3.0	400,000		
				21	71,000	4.00	8.4	55	1.2	16,000		
4	Stilb.	1	195[3]	1	15,400	7.43	14.7	95	1.0	1,200,000	Increase in cellularity and immature myeloid cells increased with decreased polymorphonuclears	Portal spaces infiltrated with lymphocytes and polymorphonuclears
				15	32,000	4.66	7.4	47	1.6	1,380,000		
				77	21,500	4.37	10.2	67	8.8	1,100,000		
				128	19,000	5.65	12.8	83	5.2	1,180,000		
10	Stilb. diprop.	5	38	1	10,000	7.64	14.4	93	0.4	1,520,000	Cellularity focally increased with clusters of immature myelocytes	Essentially normal
				11	16,000	5.39	13.5	87	0.4	129,000		
				17	44,000	6.24	12.6	82	0.2	18,000		
				38	3,700	3.19	6.3	41	1.6	338,000		
5	Stilb. diprop.	100	13	1	16,000	7.68	12.2	29	0.6	1,760,000	Moderate increase in cellularity; polymorphonuclear leucocytes decreased in number; erythropoiesis relatively decreased	Moderate fatty degeneration
				10	45,000	4.95	9.8	64	0.4	118,000		
				13	42,000	5.00	8.3	54	0	0		
14	Estradiol benzoate	3.32	13	1	8,800	6.52	12.0	78	0.4	1,708,000	Increased cellularity; immature myeloid elements predominate	Essentially normal
				8	26,000	5.04	11.1	72	3.6	705,000		
				13	41,000	4.49	11.0	71	2.4	0		
16	Estradiol benzoate	1.66	23	1	9,050	5.83	15.1	97	2.8	1,445,000	Mild hyperplasia of immature and mature granulocytes; megakaryocytes reduced; few focal areas of hypoplasia	Uniform central fatty degeneration and central necrosis
				10	18,000	4.93	12.0	78	1.8	476,000		
				20	56,000	1.75	4.0	26	0.2	0		
				23	27,800	.93	1.9	12	0	0		

[1] Accident.
[2] Distemper.
[3] Peritonitis.

no histologic abnormalities and 1 had fatty degeneration and central necrosis of the liver. In the control animals the livers were free from focal necrosis, but the hepatic cells contained a minimal amount of fat.

Male and female dogs received estradiol benzoate 1.66 mg. to 3.32 mg. by injection, or 16.32 mg. of alpha estradiol by mouth, daily. Stilbestrol was administered in doses ranging from 1 mg. to 100 mg. daily, either by mouth or parenterally. Those animals receiving the larger doses of estrogens showed no greater tendency to develop

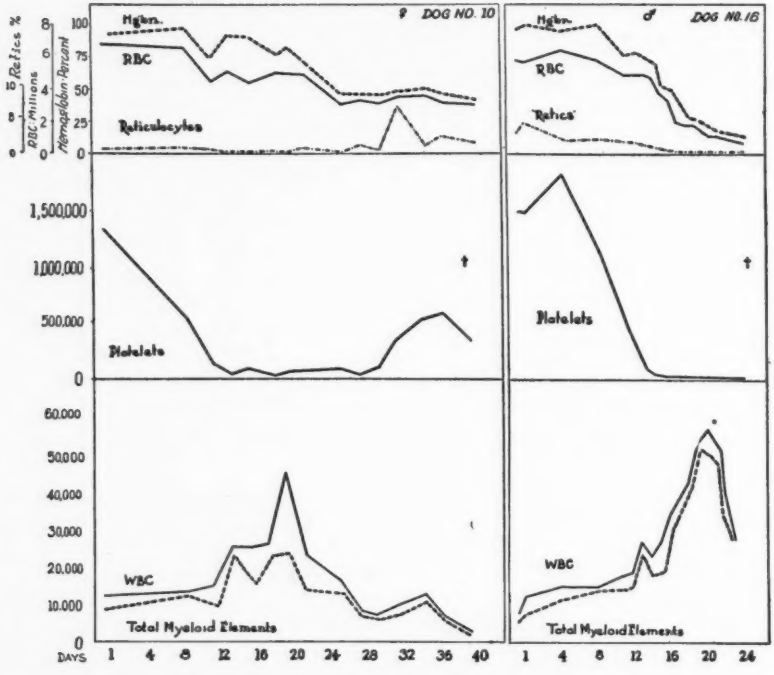

Fig. 1. Comparative effects on peripheral blood of dogs of equivalent estrogenic doses of estradiol benzoate and diethylstilbestrol dipropionate. *Dog 10* injected daily with 5 mg. stilbestrol. *Dog 16* injected daily with 1.6 mg. estradiol benzoate (table 1).

the minor hepatic changes than the ones receiving smaller doses. The values for the bromsulphthalein liver function test were consistently within normal range even when the determination was performed on animals before autopsy. The blood prothrombin estimations, icteric indices and blood calcium determinations were normal in all the dogs throughout the experiment.

The following four protocols were chosen from a total of twelve to illustrate the major changes which were observed.

PROTOCOLS

Dog 2. A male shepherd dog weighing 22.5 lb. (10 kg.) was injected daily with 10 mg. of stilbestrol dipropionate in sesame oil. The initial blood count revealed 7,300,000 erythrocytes; hemoglobin, 98% (14.4 gm.); reticulocytes 0.0%, leucocytes, 15,600, (neutrophils 50%, stabs 6%, eosinophils 7%, lymphocytes 13%, monocytes 24%) and platelets

1,500,000. The platelet count dropped concomitantly with the rise in white blood count. The final blood determinations revealed: erothrocytes 4,000,000; hemoglobin 54% (8.4 gm.), reticulocytes 1.2%, leucocytes 71,000 (neutrophils 66%, stabs 29%, myelocytes 1%, lymphocytes 2%, monocytes 2%) and platelets 16,000. This count was taken a few hours before the animal died from a sudden profuse intestinal hemorrhage. The final weight of

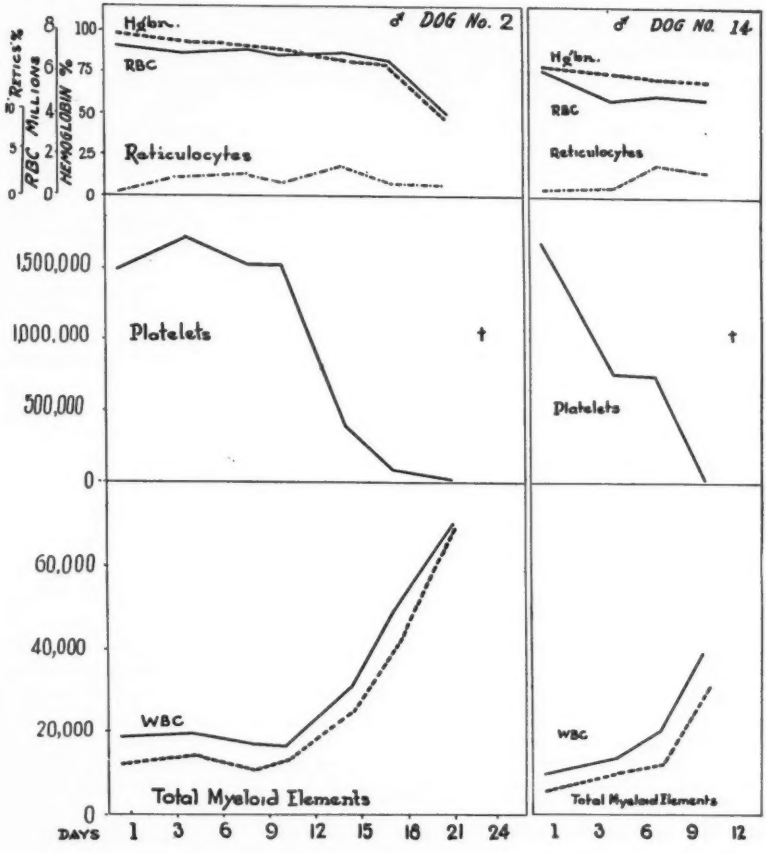

Fig. 2. Comparative effects on peripheral blood of dogs of equivalent estrogenic doses of estradiol benzoate and diethylstilbestrol dipropionate. *Dog 2* injected daily with 10 mg. stilbestrol. *Dog 14* injected daily with 3.32 mg. estradiol benzoate (table 1).

the animal was 21.5 lb. (9.77 kg.). The bromsulphthalein hepatic function test and the icteric indices were consistently within normal range. The blood prothrombin time and blood calcium determinations were normal also.

Liver. There was a uniform, moderate central necrosis and predominantly central fatty degeneration. The Kupffer cells were prominent and in many instances contained hemosiderin. *Bone marrow.* There was a slight hyperplasia of the marrow, chiefly of the myeloid series. A moderate number of polymorphonuclear cells were observed. In many instances, mature and immature cells exhibited degeneration. Erythropoiesis was not appreciably altered. Megakaryocytes were greatly reduced in number. In some areas the stroma was edematous.

Dog. 14. A male dog weighing 24 lb. (10.9 kg.) was injected intramuscularly daily with 3.32 mg. of estradiol benzoate. The initial erythrocyte count was 6,520,000, hemoglobin 78% (12.0 gm.), reticulocytes 0.4%, leucocytes 8,800 (neutrophils 56%, stabs 5%, eosinophils 5%, lymphocytes 9%, monocytes 25%) and platelets 1,708,240. This animal manifested petechiae of the mucous membranes. The final blood count taken the day before the animal died was: erythrocytes 4,940,000, hemoglobin 71% (11.0 gm.), reticulocytes 2.4%, leucocytes 41,550 (neutrophils 52%, stabs 13%, eosinophils 4%, lymphocytes 12%, monocytes 19%); the platelets were entirely absent. The animal survived thirteen days and died from an extensive subcutaneous extravasation of blood. The final weight was 24 lb. (10.9 kg.). The bromsulphthalein hepatic function test and the blood prothrombin time were normal throughout the experiment.

Liver. Essentially normal. *Bone marrow.* The marrow showed a slight increase in the degree of cellularity in which the immature myeloid cells predominated. The number of megakaryocytes was moderately reduced.

Dog 10. A female hound weighing 19.5 lb. (8.87 kg.) was injected daily with 5 mg. of stilbestrol dipropionate. Before treatment was begun the erythrocytes numbered 7,640,000, hemoglobin 96% (14.9 gm.), reticulocytes 0.4%, leucocytes 10,900 (neutrophils 66%, stabs 6%, eosinophils 1%, lymphocytes 3%, monocytes 24%) and platelets 1,520,000. By the 21st day of treatment the leucocyte count rose to 44,150 and the thrombocyte count fell to 18,720. The animal at this time had a severe gastrointestinal hemorrhage. The dog retched almost continuously and a severe loss of weight resulted. In a period of 7 days the hemoglobin dropped from 92% to 48%. The final blood studies were done on the 38th day, a few hours before the animal died. The erythrocytes numbered 3,190,000, hemoglobin 41% (6.3 gm.), reticulocytes 1.6%, leucocytes 3,700 (neutrophils 63%, eosinophils 1%, lymphocytes 1%, monocytes 35%) and platelets 338,100.

Liver. Essentially normal. *Bone marrow.* The cellularity of the marrow was focally increased with clusters of immature myelocytes predominating, but occasionally including several polymorphonuclear leucocytes. Erythrogenic foci rich in normoblasts were also present. Megakaryocytes were moderately numerous, but in several instances showed pyknosis. In several fields there was hypoplasia of the marrow and stromal edema.

Dog. 16. A male basset hound weighing 17.5 lb. (8.0 kg.) was injected daily with 1.66 mg. of estradiol benzoate. The blood picture before the experiment was begun revealed: erythrocytes 5,830,000, hemoglobin 97% (15.1 gm.), reticulocytes 2.8%, leucocytes 9,050 (neutrophils 71%, eosinophils 6%, lymphocytes 12%, monocytes 11%) and platelets 1,445,000. After a period of approximately two weeks of treatment, manifestations of bleeding began to appear. The blood picture revealed erythrocytes 4,790,000, hemoglobin 68%, (10.5 gm.), reticulocytes 0.8%, leucocytes 23,100 (neutrophils 48%, stabs 14%, eosinophils 4%, metamyelocytes 1%, lymphocytes 8%, monocytes 25%) and platelets 23,900. During the week prior to the death of the animal the platelets were consistently absent on repeated daily counts. The leucocyte count reached a peak of 56,600 and remained in this range until the day before death, at which time the white blood count dropped to 27,800 and the differential count revealed neutrophils 90%, stabs 4%, eosinophils 1%, metamyelocytes 1%, lymphocytes 1%, monocytes 3%. The erythrocyte count was 930,000 with a hemoglobin of 13% (1.9 gm.), and reticulocytes 0.0%. The animal survived 23 days, having received a total dosage of 38.18 mg. of estradiol benzoate. The bromsulphthalein hepatic function test, icteric indices, prothrombin time and the blood calcium determinations were normal on repeated occasions throughout the experiment.

Liver. There was a uniform central fatty degeneration and central necrosis. *Bone marrow.* There was a mild hyperplasia of the bone marrow, predominantly of the immature and mature granulocytes. The megakaryocytes were moderately reduced in numbers. There were a few focal areas of hypoplasia of the marrow and some stromal edema.

DISCUSSION

Equivalent doses of stilbestrol and estradiol were administered to male and female dogs. Thrombocytopenia and hemorrhage were produced in the animals which re-

ceived large doses of either of these estrogens. In the animals receiving the estradiol by injection, thrombocytopenia and hemorrhage occurred more quickly and the period of survival of these animals was shorter than that of the animals receiving comparable doses of stilbestrol. In the animals receiving the drugs by mouth, similar blood changes occurred, but they appeared later after initiation of oral treatment than after injection.

The changes in the bone marrow consisted of an increased cellularity of the myeloid elements, followed in some instances by a phase of general bone marrow hypoplasia. These changes were reflected in the peripheral blood successively by leucocytosis, thrombocytopenia, anemia, and in those animals that developed bone marrow hypoplasia, leucopenia.

The results of the liver studies were inconclusive. Although the extent of the fatty degeneration and the focal necrosis varied, the reactions in general were not marked. The fact that the necrosis was acute and not accompanied by fixed tissue proliferation would seem to indicate that it was probably of recent onset. It seems possible that the hepatic abnormalities may have been secondary to the more severe blood changes, rather than the result of direct action of estradiol or stilbestrol upon the hepatic parenchyma.

The doses of stilbestrol given to the dogs per kilogram of body weight ranged from 0.15 mg. to 12.0 mg. The doses of stilbestrol which were given to the patients ranged from 0.01 mg. to 0.11 mg. per kg. body weight. The animals therefore received the equivalent of 100 times the maximum therapeutic dose employed.

Estrogen intoxication was observed earlier and it had a more fulminating course when the hormones were administered by injection than when they were given by mouth. This may be explained by the accumulation of relatively enormous amounts of the active substance held by the oil beneath the skin of the injected animals. The dogs that were treated over a prolonged period of time with smaller doses of hormones developed chronic anemia. This is probably esplained by a gradual depression of the function of the erythropoietic tissues. Before the experiment was terminated in some animals, the peripheral red blood cell count returned to normal, which may indicate the development of greater tolerance to the estrogens by these dogs.

This study suggests that the toxic effects observed following the clinical use of the synthetic estrogen, stilbestrol, may likewise be produced by estrogens derived from natural sources. The apparent greater frequency of the toxic manifestations with the use of stilbestrol may be due to the ease with which it is administered, the high potency of the material and the fact that optimal dosages have been exceeded. Parallel clinical studies with equivalent doses of synthetic estrogens and of estrogens derived from natural sources should be undertaken.

CONCLUSIONS

Both estradiol and stilbestrol, when administered in doses much greater than those within the therapeutic range used in man, produce profound changes in the bone marrow and peripheral blood of male and female dogs.

Hepatic changes consisting of moderate fatty degeneration and central necrosis were observed following the use of both drugs. Whether these occurred as the result of direct hepatic damage or were secondary to the blood changes is uncertain.

Compared by estrogenic potency, estradiol produced more rapid and more profound changes in the bone marrow and blood than did stilbestrol.

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OBSERVATIONS ON THE EFFECT OF HYPOTHYROIDISM ON OVARIAN FUNCTION IN THE GUINEA PIG

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FOR A NUMBER OF YEARS we have been engaged in a study of functional menstrual disturbances. Our approach has been both clinical and experimental. First, the changes occurring in the genital organs and elsewhere in women suffering from these disorders have been observed and then an attempt has been made to produce comparable changes in animals by various experimental means. As a result of these studies, the following concept has been formulated.

Functional menstrual disorders may result from lesions in any of the endocrine glands or from general constitutional disease secondarily affecting the endocrine system. The primary disease condition gives rise to ovarian hypofunction and this, in turn, gives rise to the menstrual abnormality. The severity of the ovarian disturbance is indicated roughly by the endometrium. There is no constant correlation between the primary lesion, the degree of ovarian involvement, as indicated by the endometrium, and the type of menstrual disturbance.

Experimental evidence supporting the above concept has been presented previously (1, 2), and it is gratifying to note that the concept, which was first presented publicly some six years ago (3), is gradually achieving acceptance among workers in this field.

In the course of the studies referred to above, the practical value of the endometrium as an indicator of ovarian function was realized and the now popular technic of endometrial biopsy was evolved (4–6). By reason of the abundant material made available by this technic and the fact that it has been possible to produce ovarian hypofunction experimentally in animals (1, 2), it has been demonstrated that ovarian hypofunction is reflected by deviations from normal in the endometrial picture; these become progressively more marked as the ovarian disturbance increases in severity. On the basis of the type of reaction shown by the endometrium at the onset of bleeding, three levels of declining ovarian function can be clearly differentiated; these have been designated first, second and third degree ovarian failure, respectively. First degree failure is characterized by the occurrence of progestational changes in the endometrium, indicating some degree of corpus luteum activity. In second degree failure, the menstruating endometrium shows normal or excessive proliferative activity with no evidence of progestational change, indicating failure of the ovaries to secrete progesterone in amounts sufficient to produce recognizable changes in the endometrium. In third degree failure, the endometrium is atrophic or shows at best a mild proliferative reaction, indicating failure of progesterone secretion and a marked decrease in estrogen production.

First, second and third degree ovarian failure are not static or sharply delimited conditions. Each merges gradually into the next and within each category there are variations in the level of ovarian activity. The latter are indicated by variations in the intensity of the

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characteristic reaction. Transition from one level to the next is characterized by a progressive merging of the histologic characteristics of the one with those of the other.

It has been shown previously that guinea pigs subjected to partial oöphorectomy or partial hypophysectomy subsequently show abnormalities of the estrous cycle and endometrium, that the endometrial changes are comparable in every way to those which occur in association with menstrual disorders in women, that the endometrial and cyclic changes resulting from these two operative procedures are identical, and that the ovarian failure resulting from either may be of the first, second or third degree (1). Evidence regarding the effects of non-endocrine disease on ovarian function was obtained from a study of the effects of partial nephrectomy in rats (2); following this operation the animals developed hypertension and, at the same time, abnormalities of the estrous cycle identical in type with those which occur in guinea pigs as a result of partial oöphorectomy or partial hypophysectomy. Other observations which lend support to the concept stated above have been described by Dey, Fisher, Berry and Ranson (7, 8), who found that guinea pigs with lesions in the hypothalamus show changes in the reproductive system indicative of first, second or third degree ovarian failure, presumably secondary to pituitary dysfunction.

Our own investigation has been extended to include a study of ovarian function in thyroidectomized guinea pigs. The purpose of the present communication is to present the results of this study.

METHODS

Thirty-six adult female guinea pigs were thyroidectomized. Subsequently, the estrous cycles were observed for periods varying from 57 to 138 days. The metabolic rate was determined at intervals before and after operation, and body weights were recorded at the time of operation and at 3-day intervals thereafter. Sections of the uterus were removed from 11 animals during the experimental period. At the end of the observation period, all animals were killed and examined for the presence or absence of thyroid tissue. The ovaries were weighed, and the ovaries, uteri and vaginae were removed and prepared for microscopic study. Biopsy and autopsy were timed in relation to the estrous cycle as indicated in figures 2 and 3. Approximately half of the specimens were removed during the interval corresponding to the height of the lutein phase (days 4 to 9 of the cycle), a few were removed later in diestrus, the remainder during estrus.

Thyroidectomy was performed in the following manner. The animal was anesthetized with ether and the operative site was shaved. Incision approximately 4 cm. long was made through the skin in the midline from a point just above the larynx to the level of the sternoclavicular junction. By further sharp and blunt dissection the trachea was exposed. The thyroid gland was easily identified just lateral to the trachea and larynx, with a small isthmus connecting the two lobes (this isthmus was sometimes absent). By careful inspection and blunt dissection of the surrounding loose areolar tissue, the small (1 to 2 mm.) parathyroid glands (one, sometimes two, on each side) could be identified and left in situ while the thyroid was removed. The upper and lower poles of each lobe were carefully dissected free and the blood supply to each pole was ligated with silk in such a manner as to allow removal of the entire gland. Great care was taken not to injure the laryngeal nerve. Bleeding was controlled with additional silk ligatures when necessary. The fascia and skin were closed with continuous silk suture. With this technic, no animals died of parathyroid tetany.

The metabolic rate, or rate of oxygen consumption, was determined satisfactorily with the apparatus illustrated in figure 1. With this method the oxygen consumption rate of any one animal could be determined from time to time. The unit used for comparison was the number of cc. of oxygen consumed per 100 gm. of body weight per hour, corrected to standard conditions. Four determinations were made on each

animal prior to thyroidectomy and from 2 to 5 determinations subsequent to the operation. Each postoperative reading was compared with the average of the four preoperative readings from the same animal. Thus, actual variations in oxygen consumption rate in single individuals were observed, not variations from a fixed standard.

In the guinea pig the vaginal opening is closed during diestrus by a solid cord of epithelium which binds the dorsal and ventral lips of the vestibule together. With the

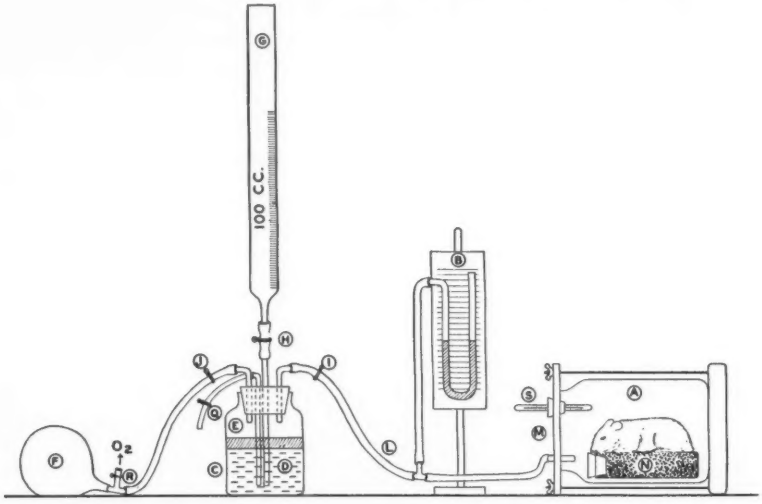

Fig. 1. Apparatus used for determining the rate of oxygen consumption. An animal is placed in the glass chamber (A) which contains soda lime in wire baskets (N). The animal is then allowed to remain quiet for a rest period of 5 or 10 minutes before the lid of the animal chamber is fixed air-tight. The rubber balloon (F) is filled with oxygen through R and with all stop-cocks closed except J and Q, oxygen is allowed to run into bottle (E), forcing water out through Q. Stop-cocks Q and J are then closed, and stop-cock I is opened, which allows oxygen into the animal chamber through L. As the oxygen is consumed in A, the pressure is decreased, which is shown by the manometer (B). By adding water to D from the burette (G), the oxygen from E is displaced into A, where it is consumed. With the system closed and the animal at rest, and more than 100 cc. of oxygen in the bottle at E, the time is checked when the manometer shows the pressure to be the same in E and A. One hundred cc. of water is then allowed to run into D gradually, thus displacing 100 cc. of oxygen into A. When the pressure is again the same, the time is again checked; thus the time required for the consumption of 100 cc. of oxygen is determined. The volume of oxygen is reduced to standard conditions from local atmospheric pressure and the temperature inside the animal chamber (recorded by thermometer S). By closing I and opening Q, the water (D) may be displaced through Q and the operation repeated without removing the animal from the chamber.

approach of estrus this epithelial cord is dissolved and communication between the vaginal lumen and the exterior is established. The vagina remains open during estrus but is closed again in early metestrus. In the present experiments, the cycles were studied by examining the animals once daily and obtaining vaginal smears (once daily) whenever the membrane was open.

The ovaries, uteri and vaginae were fixed in Bouin's solution, embedded in paraffin, sectioned at 6 μ and stained with hematoxylin and eosin. Serial sections of both ovaries and representative sections of the uterus and vagina of each animal were studied microscopically.

Using the same methods employed in the study of the thyroidectomized animals, the following control observations were made on intact guinea pigs from the same

colony, maintained under the same conditions of environment and diet as were the thyroidectomized group.

(*a*) The estrous cycles of 109 animals were observed for periods varying from 40 to 310 days (table 1). Body weights were recorded at 4-day intervals throughout the period of observation.

(*b*) Sections of uterus were removed from 20 of the above animals and 13 of these animals and 6 others of the above group were killed at the end of the observation period. The ovaries were weighed at autopsy and the ovaries, uteri and vaginae removed and prepared for histologic study. Serial sections of both ovaries and representative sections of the uterus and vagina of each animal were studied microscopically.

TABLE 1. OBSERVATIONS ON CYCLES OF CONTROL GUINEA PIGS

Type of cycle	Length of observation period	No. guinea pigs	Total No. guinea pigs
	days		
Comparable to that of *guinea pig 244*	40– 60	26	
	70–100	11	
	120–310	20	57
Comparable to that of *guinea pigs 253* and *271*[1]	40– 65	6	
	70–100	16	
	110–310	16	38
Comparable to those of *guinea pigs 246* and *236*	50– 60	2	
	70– 90	7	
	160–320	5	14

[1] In 35 of these, the cycles were comparable to that of *guinea pig 244* after the body weight reached 400 gm.

All of the tissues from this group of animals were removed during estrus or during the height of the lutein phase (days 4 to 9 of the cycle).

(*c*) Serial sections of one or both ovaries from 23 guinea pigs killed at various times during the estrous cycle were studied microscopically. Each stage of the cycle was represented by several specimens.

(*d*) One control animal was subjected to a sham operation. No abnormalities were observed in the cycles or genital tissues of this animal.

All observations prior to and at autopsy were made by one of us; the tissues were studied by the other two. Ovaries, uteri, vaginae and cycles were studied separately, without reference to the history of the animal or to any other set of findings. Observations on the ovaries, uterus, vagina and cycle of each animal were then correlated and the animals were classified on the basis of these data. Finally, the observations on the reproductive system were correlated with those relating to thyroid function.

RESULTS AND DISCUSSION

Observations on the vaginal cycles of the control animals are summarized in table 1. No variations from the normal picture, as described in the literature (9–13), were observed in the cycle, ovaries or endometria of the control animals.

The oxygen consumption rate of different animals prior to thyroidectomy ranged from approximately 65 to approximately 80 cc. per 100 gm. body weight per hour. These figures are the averages, respectively, of the minimum and maximum pre-operative readings from all of the animals. There was little variation from time to time in the pre-operative rate of each animal. In the majority of cases the rate decreased following thyroidectomy (fig. 2, 3). However, the postoperative rate was not

considered subnormal unless it fell below 65 cc. per 100 gm. per hour, as well as below the animal's own pre-operative average.

The B.M.R. of some animals decreased temporarily after thyroidectomy and subsequently returned to or toward the pre-operative level. A reason for changes of this

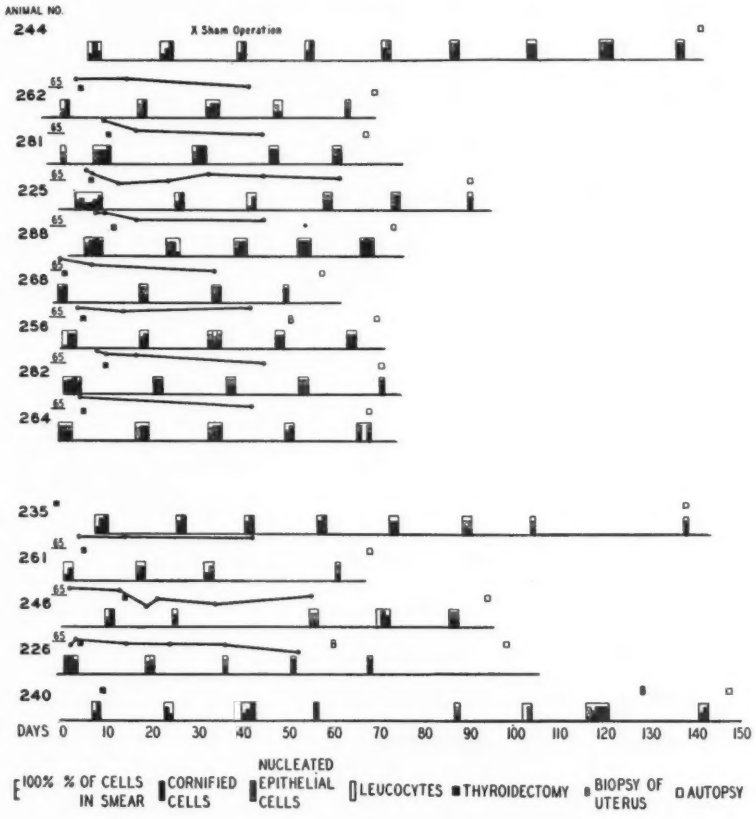

Fig. 2. Records of vaginal cycles and variations in B.M.R. of a control animal and of experimental animals in which grossly visible thyroid tissue was found at autopsy. In the records of the cycles, absence of symbols indicates vagina closed. The record of *guinea pig 244* illustrates the typical normal record. The record of *guinea pig 262* illustrates the findings in 6 experimental animals whose records are not shown. The changes in oxygen consumption rate after thyroidectomy are shown by the line graphs. The base line for each graph is the base line of the corresponding cycle record. The graph is drawn on a scale of 10 units, 1 unit equalling 5 cc. of oxygen consumed per 100 gm. body weight per hour under standard conditions. The average of the minimum pre-operative readings from all guinea pigs is indicated by the figure 65. Where line graphs are lacking, there was no decrease in B.M.R. after thyroidectomy.

type was apparent at autopsy. It should be noted that all visible thyroid tissue was removed from every animal operated upon. At autopsy, hypertrophied aberrant thyroid tissue, macroscopic fragments or a completely regenerated gland was found in 19 animals. In 17 guinea pigs no thyroid tissue or only a microscopic fragment was

found.[1] The B.M.R. was normal or only slightly subnormal in the animals in which grossly visible thyroid tissue was found, while it was more than 10% below normal in 10 of the 17 animals in which thyroidectomy was complete or nearly so (table 2).

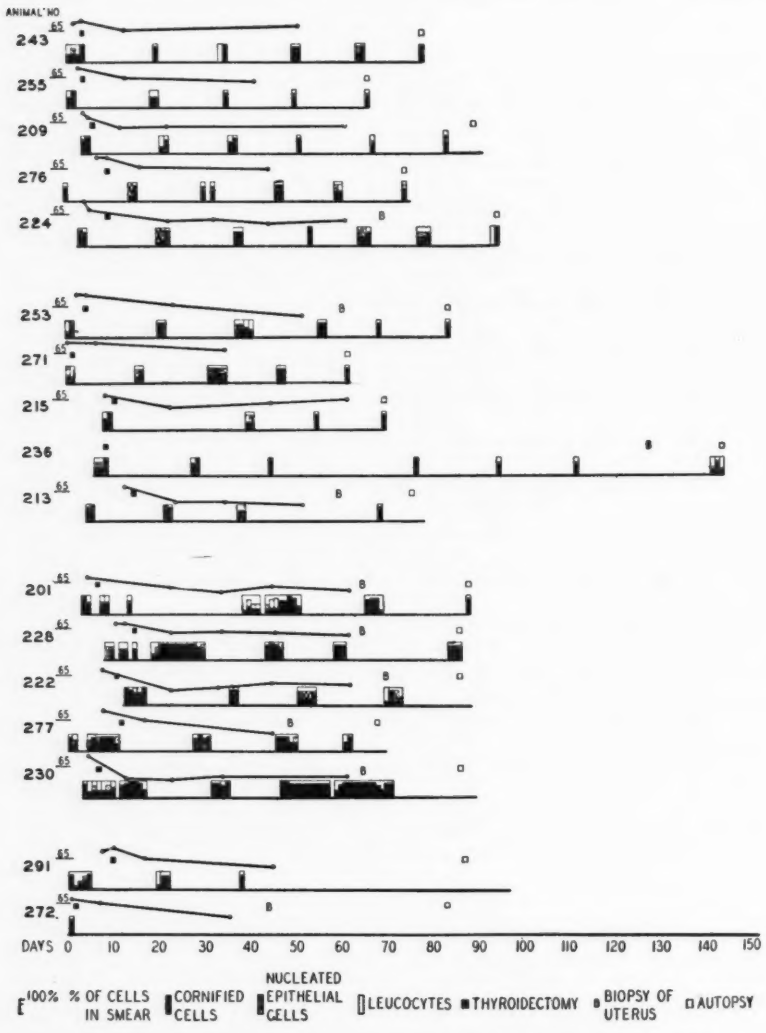

Fig. 3. Records of vaginal cycles and variations in B.M.R. of thyroidectomized guinea pigs. See Figure 2 for explanation of graphs.

These findings indicate that aberrant thyroid tissue is of common occurrence in the guinea pig, that the regenerative power of the guinea pig thyroid is remarkable and

[1] For the sake of brevity, these 17 animals were classified as completely thyroidectomized. Actually, microscopic fragments of the gland were found in *guinea pigs 215, 213, 236, 271* and *228*. In the remaining 12 animals of this group, no thyroid tissue was found.

that the regenerated gland is capable of maintaining an approximately normal B.M.R.

Observations on the vaginal cycles are shown in figures 2 and 3. Subsequent to thyroidectomy, 19 animals continued to exhibit normal cyclic rupture of the vaginal closure membrane and cornification of the vaginal smear at intervals of approximately 16 days; 10 animals showed a predominance of normal cycles with occasional minor abnormalities; 5 showed prolonged periods of estrus; and in 2 animals the vaginal closure membrane remained closed throughout the greater part of the experimental

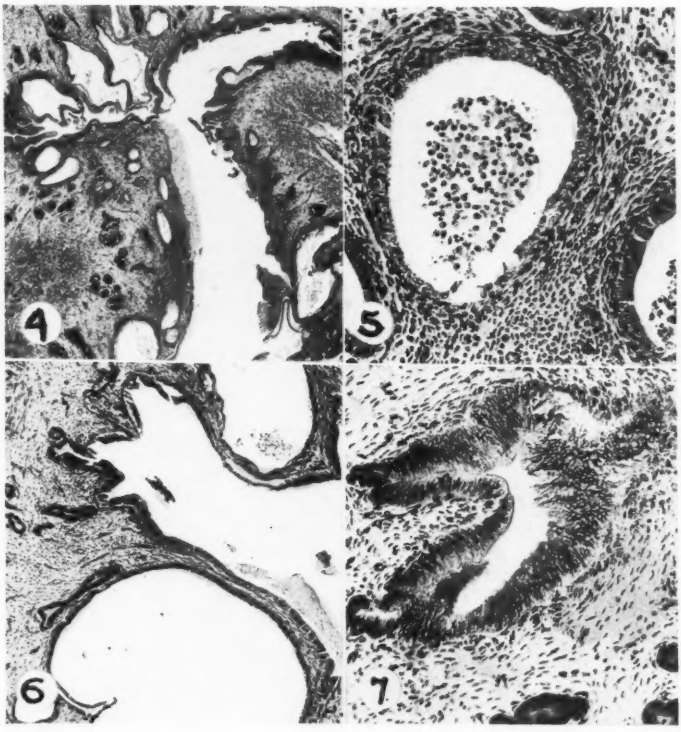

Fig. 4. Section of uterine biopsy from *guinea pig* 222. ×22, Fig. 5. Section of uterine biopsy of *guinea pig* 277. ×120, Fig. 6. Section of uterine biopsy of *guinea pig* 230. ×46, Fig. 7. Section of uterine biopsy of *guinea pig* 230. ×120.

period. All of the animals exhibiting severe abnormalities of the cycle were completely thyroidectomized and in all of them the B.M.R. was at least 10% below normal.

In 20 cases the endometrium showed no definite abnormalities. In 12 cases it showed mild abnormalities of various types and in 4 cases it showed glandular cystic hyperplasia (fig. 4–7). The picture in the latter specimens was comparable to glandular cystic hyperplasia of the human endometrium. The reaction was characterized by marked proliferative activity of the surface and glandular epithelium, variation in gland size, and cystic dilatation of some glands. The surface epithelium and the epithelium of the non-dilated glands was markedly pseudostratified and showed many mitotic figures. The epithelium of the dilated glands was pseudostratified, cuboidal or

TABLE 2. CORRELATION BETWEEN AMOUNT OF THYROID TISSUE PRESENT AND BASAL OXYGEN CONSUMPTION RATE IN GUINEA PIGS

Thyroid[1]	No. of animals with B.M.R. as indicated, after thyroidectomy			Totals
	Normal	Below normal		
		2%–10%	15%–25%	
Present	13	6	0	19
Absent	4	3	10	17
Totals	17	9	10	36

[1] Autopsy findings.

flattened. The stroma was loose and edematous; its cells had small nuclei and sparse cytoplasm. The reaction in these specimens was clearly an exaggeration of the normal estrous reaction and it was similar in every respect to the condition produced in the castrate guinea pig endometrium as a result of prolonged treatment with estrogen (14–16).

The ovaries of 24 animals were essentially normal histologically; those of 6 guinea pigs showed evidence of temporary or prolonged failure of corpus luteum formation, and those of 6 animals showed features suggestive of subnormal corpus luteum formation. No marked abnormalities of the follicles were noted. The ovarian weights were slightly subnormal in a few but in most instances they were within normal limits.

The findings in the individual animals are summarized in the protocols and the correlations between the B.M.R. and conditions in the reproductive system are shown in tables 3 and 4. Reference to the protocols reveals that 11 guinea pigs showed no abnormalities in the reproductive system, 6 showed conditions of questionable sig-

TABLE 3. RELATIONSHIP BETWEEN AMOUNT OF THYROID TISSUE PRESENT, B.M.R. AND CONDITIONS IN REPRODUCTIVE SYSTEM OF GUINEA PIGS

Thyroid	B.M.R.	No. animals	Conditions in reproductive system			Genital abnormalities	
			No. normal	No. atypical	No. abnormal	No. mild	No. marked
Present	Normal	13	6	1	6	6	0
Absent	Normal	4	1	1	2	1	1
Present	2%–10%	6	3	3	0	0	0
Absent	below normal	3	1	0	2	1	1
Absent	15%–25% below normal	10	0	1	9	3	6
Present	Normal to 25%	19	9	4	6	6	0
Absent	below normal	17	2	2	13	5	8
Present or Absent	Normal	17	7	2	8	7	1
	2%–10% below normal	9	4	3	2	1	1
	15%–25% below normal	10	0	1	9	3	6
Totals		36	11	6	19	11	8

TABLE 4. RELATIONSHIP BETWEEN OVARIAN FUNCTION AND BASAL OXYGEN CONSUMPTION RATE IN GUINEA PIGS

B.M.R.	Animals showing the conditions indicated in the reproductive system												Totals
	Normal		Cycle normal, other findings abnormal		Cycle atypical, other findings normal		Cycle abnormal (mild), other findings abnormal (mild)		Cycle abnormal (severe), other findings abnormal (mild)		Cycle abnormal (severe), other findings abnormal (severe)		
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Normal	7	41.1	6	35.3	2	11.7	2	11.7	0	00.0	0	00.0	17
2%–10% below normal	4	44.4	1	11.1	3	33.3	0	00.0	1	11.1	0	00.0	9
15%–25% below normal	0	00.0	1	10.0	1	10.0	2	20.0	3	30.0	3	30.0	10
Totals	11		8		6		4		4		3		36

nificance, 7 showed minor but distinct variations from normal, and 12 showed more severe abnormalities. Four of the latter group showed marked abnormalities for a short period of time with subsequent reversion toward normal, and 8 showed marked abnormalities for more prolonged periods.

As shown in tables 3 and 4, there was a close correlation between the degree of hypothyroidism, as indicated by the B.M.R., and the severity of the abnormalities in the reproductive system. The data recorded in these tables afford evidence that the reproductive abnormalities observed were actually due to hypothyroidism.

There was no constant correlation between the endometrial picture and the type of vaginal cycle in these animals, although there was a general tendency toward parallelism between the degree of abnormality in the cycle and in the endometrium. With respect to evidence concerning the level of ovarian activity, there was close

TABLE 5. CORRELATION BETWEEN ENDOMETRIAL FINDINGS, VAGINAL CYCLE AND CORPORA LUTEA IN OVARIES OF GUINEA PIGS

Type of animal	Endometrial findings	Vaginal cycle	No. animals	No. of corpora lutea[1]		
				Min.	Max.	Av.
Thyroidectomized	Normal	Normal	13	4	13	7.6
		Mild abnormalities	6	5	11	8.1
	Mild abnormalities	Normal	5	4	8	6.2
		Mild abnormalities	4	4	8	5.5
	Mild abnormalities	Prolonged diestrus	2	4	7	5.2
		Prolonged estrus	2	4	5	4.5
	Hyperplastic	Normal	1	7	7	7.0
		Prolonged estrus	3	0	3	2.0
Control	Normal	Normal	11	6	11	8.1

[1] All corpora lutea of the 3 ovulation periods preceding autopsy were counted. These 3 generations of corpora lutea can be identified in the ovaries of normal guinea pigs autopsied during the lutein phase. If guinea pigs autopsied in estrus had not yet ovulated, normal pre-ovulatory follicles and the corpora lutea of the 2 preceding ovulations were counted.

agreement between the ovarian and endometrial findings and even closer agreement between the ovarian picture and the evidence afforded by the endometrium and vaginal cycle combined. This is illustrated by the observations summarized in table 5. The abnormalities in the reproductive system were indicative of disturbances of ovarian function varying in different animals from mild partial failure of corpus luteum formation to complete absence of corpus luteum activity for short or prolonged periods, or, in other words, of ovarian hypofunction of the first or second degree. The results of the experiments afford evidence, therefore, that the abnormalities in the endometrium and vaginal cycle which result from hypothyroidism are due to ovarian hypofunction and that the degree of hypofunction is reflected with considerable accuracy by the endometrium.

SUMMARY AND CONCLUSIONS

Adequately controlled observations were made on the oxygen consumption rate, estrous cycles, ovaries, endometria and vaginae of 36 thyroidectomized guinea pigs. The cycles and B.M.R. were observed for periods of 57 to 138 days. It was found that complete removal of the thyroid is a difficult procedure in the guinea pig, because of the remarkable regenerative power of the gland and the frequent occurrence of aberrant thyroid tissue. The regenerated gland is capable of maintaining the animal in a relatively normal state or with only slight deviations in the B.M.R., endometrium and vaginal cycle. In those cases in which the gland did not regenerate, an occasional animal maintained a normal or fairly normal estrous cycle but the majority of the animals tended to show significant deviations in the B.M.R., vaginal cycle and endometrium. From an analysis of the conditions observed in the reproductive system and a correlation of these with the findings relating to thyroid function, the following conclusions regarding the effects of hypothyroidism on ovarian function in the guinea pig can be drawn.

Hypothyroidism may give rise to disturbances of ovarian function.

The degree of true primary hypothyroidism necessary to produce significant changes in ovarian function is severe.

The changes which are produced may vary in severity in different individuals.

All cases of ovarian hypofunction resulting from hypothyroidism in the guinea pig tend to be of the first or second degree.

In guinea pigs with hypothyroidism, the level of ovarian activity is reflected with considerable accuracy by the endometrium.

In those cases in which the hypothyroidism gives rise to disturbances of ovarian function, there is no constant correlation between the resulting endometrial picture and the type of cyclic activity in the vagina.

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PROTOCOLS

Group 1. The cycles of the animals in this group were normal and no abnormalities were noted in the ovaries, endometria or vaginae. In each case the endometrium and vagina showed the reaction which is typical for the stage of the cycle indicated by the ovarian histology.

Guinea pigs 225, 239, 249, 262, 258, 281. Thyroid was present, B.M.R. normal. *Guinea pigs 225* and *249* were autopsied in estrus; the ovaries of *225* contained freshly ruptured follicles, those of *249* contained corpora lutea at a stage of development corresponding to about day 3 of the normal cycle.

Guinea pigs 286, 294, 290. Thyroid was present, B.M.R. 2% to 10% below normal. The ovaries of *guinea pig 286*, autopsied in estrus, contained normal follicles in the late preovulatory stage of development.

Guinea pig 243. Thyroid was absent, B.M.R. normal, autopsy day 1 of the cycle.

Guinea pig 255. Thyroid was absent, B.M.R. varied from 2% to 10% below normal, autopsy day 1 of the cycle. The ovaries of both animals contained normal follicles in the early pre-ovulatory stage of development.

Group 2. The ovaries, endometria and vaginae of the animals of this group were normal. The records of the cycles showed minor variations from the typical normal picture. Similar variations were noted in a few of the control animals (table 1); therefore the variations observed in the experimental animals are of questionable significance. For convenience in tabulating observations, the reproductive findings in these animals were classified as atypical.

Guinea pig 261, thyroid was present, B.M.R. normal, *guinea pig 235*, thyroid was present, B.M.R. 2% below normal, autopsy day 1, ovaries contained freshly ruptured follicles, *guinea pig 246*, thyroid was present, B.M.R. 10% below normal.

Guinea pig 271. Thyroid was absent, B.M.R. normal, autopsy day 1 of the cycle. The ovaries contained freshly ruptured follicles. The endometrium showed the reaction characteristic of metestrus. It contained one cystic gland which occupied the whole width of the stroma. While an occasional cystic gland may be seen during metestrus in normal animals, the degree of dilatation is not usually as extreme as in this case. The vagina was cornified. In all other cases (6) in which autopsy was performed at this stage of the ovarian cycle, the vagina showed the picture typical of metestrus.

Guinea pig 226. Thyroid was present, B.M.R. 5% to 8% below normal, autopsy 30 days after estrus, vagina closed. The ovaries contained corpora lutea at the stage of development typical of day 3 of the normal cycle. The endometrium and vagina showed the reactions typical of metestrus. An endometrial biopsy removed on the 10th day of cycle showed no abnormality.

Guinea pig 215. Thyroid was absent, B.M.R. 10% to 15% below normal, autopsy 30 days after estrus, vagina closed. The ovaries contained freshly ruptured follicles. The endometrium and vagina showed reactions typical of metestrus.

Group 3. The significance of the reproductive findings in this group of animals could not be determined with certainty on the basis of the data at hand. The records of the cycle were similar to those of normal animals or to those of the animals in *group 2*. In the endometria there were distinct variations from the normal picture as described in the literature (9–13), and as observed in our own series of controls. Moreover, conditions similar to those observed in these animals were noted in other animals showing definite abnormalities in the genital tract. Therefore, for the sake of convenience in tabulating observations, the conditions in these animals were classified as abnormal.

Guinea pigs 253 and *224.* Thyroid was absent, B.M.R. 20% below normal, autopsy day 1 of the cycle. The ovaries of both animals contained freshly ruptured follicles. The endometrial specimens removed at autopsy were normal, although that of *guinea pig 224* showed rather poor glandular development, and the vaginae showed the normal metestrous picture. The endometrial biopsies, removed in each case on the 6th day of the cycle, showed some evidence of corpus luteum activity. However, the picture was entirely unlike that seen in any specimen removed from a normal animal during the lutein phase. It was suggestive of a low level of corpus luteum activity.

Guinea pig 240. Thyroid was present, B.M.R. normal. A specimen of endometrium removed 13 days after opening of the vagina, and 8 days after the vagina closed, showed a reaction comparable to that typical of the height of the lutein phase. The endometrial reaction and the apparent age of the corpora lutea of this cycle suggested that ovulation occurred about 2 or 3 days after the vagina closed. The endometrial specimen removed at autopsy, and likewise the ovaries and vagina, were essentially normal. The ovaries contained corpora lutea of the recent ovulation and a small lutein body containing an ovum. The lutein cells composing the latter body were similar in appearance to those of the corpora lutea. No

single finding in this animal could be considered abnormal but the complete picture was suggestive of abnormality.

Guinea pig 213. Thyroid was absent, B.M.R. 22% below normal. The autopsy findings were essentially normal but the total number of corpora lutea was below the normal range. There were 4 corpora lutea of the recent ovulation, one each of the two preceding ovulations. An endometrial biopsy removed late in diestrus showed the general characteristics of the lutein phase, with a fair number of mitotic figures in the stroma. However, the stroma was much more compact than normal and the stromal cells smaller than normal, with less abundant cytoplasm.

Guinea pig 264. Thyroid was present, B.M.R. normal, autopsy 3rd day of the cycle, vagina open. In one ovary there was a corpus luteum at the stage of development characteristic of day 4 of the normal cycle. The other ovary contained 2 corpora lutea which appeared to be about 24 hours younger and a luteinized follicle slightly smaller than the corpora lutea but similar to them in appearance except for the presence of the ovum. The vagina showed the normal metestrous picture. With respect to gland pattern and stroma reaction, the picture in the endometrium was typical of metestrus. However, the epithelial structures did not show the regressive changes characteristic of metestrus, but rather, a marked proliferative reaction. Mitotic figures were abundant in the surface epithelium and in the epithelium of the necks and fundi of the glands. Normally, the early lutein phase is characterized by some mitotic activity in the epithelium of the basal portions of the glands but mitotic figures have disappeared from the surface epithelium, or nearly so, before they appear in any abundance in the basal portions of the glands.

Guinea pig 282. Thyroid was present, B.M.R. normal, autopsy day 1 of the cycle. The ovaries were normal histologically. They contained corpora lutea showing the stage of development characteristic of about day 4 of the normal cycle. The vaginal reaction was similar to that characteristic of the lutein phase of the cycle. The endometrium showed excessive proliferation of the surface and glandular epithelium similar to that seen in *guinea pig 264.* The endometrial reaction normally associated with corpora lutea showing the stage of development seen in this animal is characterized by mitotic activity in the stroma, absence of mitotic activity in the surface epithelium, and in the upper portions of the glands. The stroma in this case showed only an occasional mitotic figure.

Guinea pig 236. Thyroid was absent, B.M.R. normal, autopsy day 2 of the cycle. The ovaries were normal histologically. They contained corpora lutea at about the same stage of development as those in the ovaries of *guinea pig 282.* The endometrium and vagina showed the typical lutein phase reaction; the endometrial glands were poorly developed. An endometrial biopsy removed 30 days after estrus showed a picture similar to that seen in *guinea pig 264,* with even more excessive epithelial proliferation.

Group 4. Guinea pig 228. Thyroid was absent, B.M.R. 10% below normal, autopsy day 3 of the cycle, vagina open. The ovaries were normal histologically. They contained corpora lutea at about the same stage of development as those in the ovaries of the two preceding animals. The endometrium showed excessive proliferation of the surface and glandular epithelium. The vagina showed a picture comparable to that characteristic of late diestrus (mucification). An endometrial biopsy removed on the 7th day of a prolonged cycle showed evidence of corpus luteum activity (mitoses in the stroma and typical gland pattern), but the picture was definitely abnormal. The stromal cells were small, with sparse cytoplasm and there was marked edema.

Guinea pig 272. Thyroid was absent, B.M.R. 20% below normal, no vaginal estrus after thyroidectomy. The ovaries contained two corpora lutea showing the stage of development characteristic of about days 4 to 5 of the normal cycle, one spurious corpus luteum similar in appearance to the true corpora except for the presence of an ovum, 3 corpora which appeared to be about 15 to 20 days old and one very old corpus luteum. The endometrium showed excessive proliferation of the surface and glandular epithelium similar to that seen in *guinea pig 264* but of more marked degree. An endometrial biopsy removed 37 days before autopsy showed a weak follicular phase reaction. The picture was comparable to that produced in the castrate guinea pig endometrium as a result of prolonged treatment with subcornifying doses of estrogen (1.0 I.U. daily) (17).

Guinea pig 276. Thyroid was absent, B.M.R. normal, autopsy day 1 of the cycle. The ovaries were normal histologically. They contained 2 normal and 3 atretic follicles in the late preovulatory stage of development and two sets of degenerating corpora lutea. The corpora lutea of the more recent ovulation were in a somewhat more advanced stage of degeneration than is characteristic for corpora lutea 15 to 16 days old. The vagina was cornified. The endometrium showed glandular cystic hyperplasia. In one area the stroma was compact, non-edematous and richly cellular. In this area the stromal picture was similar to that seen in *guinea pigs 268* and *256.* Elsewhere the stroma presented the appearance characteristic of endometrial hyperplasia.

Guinea pig 291. Thyroid was absent, B.M.R. 18% below normal, autopsy in prolonged diestrus. The ovaries contained 1 normal and several atretic follicles in the late pre-ovulatory stage of development, one corpus luteum approximately 16 days old, and another only half as large but showing about the same degree of cellular degeneration. There was also one very old corpus luteum. The endometrium showed the

reaction characteristic of estrus, with marked proliferation of the epithelium, especially in the upper one-third of the glands, and excessive edema. The vagina showed a normal proestrous reaction.

Guinea pig 201. Thyroid was absent, B.M.R. 18% to 20% below normal, autopsy day 1 of the cycle. The ovaries contained 2 young corpora lutea, 1 corpus luteum apparently 15 to 16 days old, and one very old corpus luteum. The endometrium and vagina showed the normal metestrous picture. An endometrial biopsy removed one day before estrus showed the reaction characteristic of proestrus, with marked edema and some glandular dilatation.

Guinea pig 209. Thyroid was absent, B.M.R. 10% below normal, cycle normal, *guinea pig 288*, thyroid present, B.M.R. normal, cycle normal, *guinea pig 268*, thyroid present, B.M.R. normal, cycle normal, *guinea pig 256*, thyroid present, B.M.R. normal, cycle normal. The above animals were autopsied during the lutein phase of the cycle. The ovaries contained corpora lutea of the recent ovulation and corpora lutea in a very advanced stage of degeneration, but no corpora lutea of the previous cycle. The vaginae were normal. The endometria of *guinea pigs 209* and *288* were normal. The endometria of *guinea pigs 268* and *256* showed the general picture characteristic of the lutein phase. The picture varied from normal in that the stroma was very compact and exceedingly rich in cells, the capillary network was less highly developed than normally and there were few mitoses in the stromal cells. An endometrial biopsy removed from *guinea pig 256* on the 4th day of the cycle for which corpora lutea were lacking showed excessive proliferation of the surface and glandular epithelium similar to that seen in *guinea pigs 264, 282, 236, 228* and *272*.

Guinea pig 277. Thyroid was absent, B.M.R. 20% to 22% below normal, autopsy day 8 of the cycle. The ovaries contained young corpora lutea but no degenerating corpora lutea. The endometrial picture was similar to that seen in the endometria of *guinea pigs 268* and *256*. The vagina showed the normal lutein phase reaction. An endometrial biopsy removed during prolonged estrus showed typical glandular cystic hyperplasia (fig. 5).

Guinea pig 222. Thyroid was absent, B.M.R. 12% to 15% below normal, autopsy 17 days after vagina opened and 13 days after the vagina closed. The ovaries contained follicles in the pre-ovulatory stage of development showing early atresia. There were no normal follicles. There was one spurious corpus luteum showing early degenerative changes. It was similar in size and, except for the presence of an ovum, in appearance, to a true corpus luteum. The vagina was mucified, showing a picture similar to that characteristic of about day 15 of the normal cycle. An endometrial specimen removed during prolonged estrus showed typical glandular cystic hyperplasia (fig. 4). The endometrial specimen removed at autopsy also showed hyperplasia but the degree of proliferative activity in the epithelium was less marked and the stroma was more compact and less edematous.

Guinea pig 230. Thyroid was absent, B.M.R. 18% to 25% below normal, autopsy 15 days after the end of a period of prolonged estrus. The ovaries contained no corpora lutea, no large follicles and no normal growing follicles. There was a fair number of small to medium-sized follicles showing advanced atresia. The vagina was mucified, showing a picture comparable to that characteristic of about day 11 of the normal cycle. An endometrial biopsy removed during prolonged estrus showed glandular cystic hyperplasia with areas of necrosis (fig. 6–7). The picture was comparable to that produced in the castrate guinea pig endometrium by prolonged treatment with 6.5 I.U. of estrogen daily (11, 12). The endometrial specimen removed at autopsy also showed endometrial hyperplasia.

THE EFFECT OF ESTROGEN ON THE PERMEABILITY OF THE UTERINE CAPILLARIES[1]

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FOR SOME TIME it has been known that estrogens produce fluid accumulation in the uterus of experimental animals. Although the length of the latent period following estrogen administration (1) and the electrolytic shifts involved (2) have been evaluated, the mechanism by which the edema reaction proceeds is obscure. In general, the principal factors which favor enhanced fluid accumulation have been listed as follows (3): (*a*) increases in the capillary pressure, (*b*) increases in the permeability of the capillary membranes, (*c*) reduction in the osmotic pressure of plasma as compared to tissue fluid, (*d*) obstruction of the lymph channels. To produce uterine edema, estrogen must set into motion one or more of the above factors or some new mechanism hitherto not described. In an attempt to elucidate the mechanism of the uterine fluid accumulation induced by estrogen, we have tried to evaluate, in part, the effect of estradiol benzoate on the factors producing edema. To this end we have studied the effect of estrogen on the permeability of the uterine capillaries to trypan blue.

METHODS AND RESULTS

Menken has recently reviewed the use of dyes in the study of changes of capillary permeability (4). There seems to be ample evidence to indicate that trypan blue accumulates in areas where the capillary permeability is increased. To follow changes in the permeability of the capillaries of the uterus we studied the uterine accumulation of trypan blue intravenously administered. The amount of blue in the tissue after injection depends upon the amount of dye within the vascular system and within the tissue proper. Since the distribution of the dye accumulated can be ascertained by histological procedures, it is possible to determine the amount of trypan blue taken up by the tissue independent of changes in the vascular channels.

Ten rats castrated approximately two weeks prior to the date of the experiment and weighing between 150 and 200 gm. were divided into 2 groups. One group received a single injection of 100 I.U. estradiol benzoate intramuscularly; the remaining rats were given sesame oil by the same route. Forty-seven and one-half hours after the estrogen injection, the rats were anesthetized with avertin (1 cc. of 2.5% solution /100 gm. body weight). One jugular vein was exposed and trypan blue (1.0 cc. of a 1% trypan blue solution in saline per 100 gm. body weight) was injected directly into that vessel. Exactly 30 minutes after the dye injection the animals were killed by an

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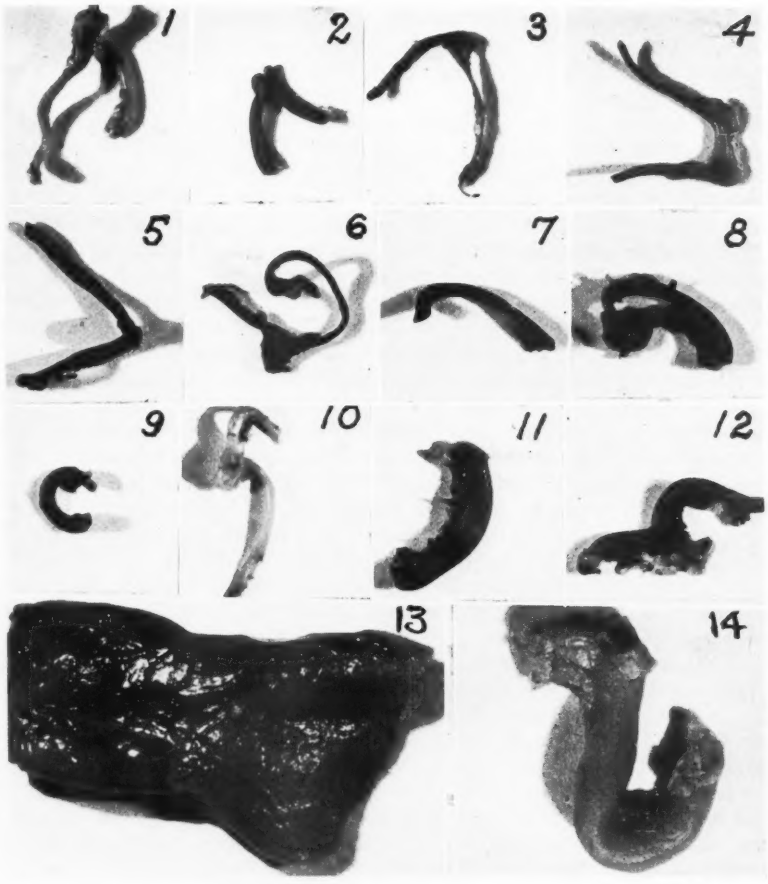

Figs. 1–14.—THE MACROSCOPIC APPEARANCE OF REPRESENTATIVE RAT UTERI AFTER TRYPAN BLUE INJECTIONS IN VARIOUS GROUPS. The degree of trypan blue intake is proportionate to the shading in the photograph. (1) castrate control, (2) castrate which received 1½ times the original dosage of the dye, (3) castrate receiving twice the average dosage, (4) estrogen 1 hour, (5) estrogen 2 hours, (6) estrogen 3 hours, (7) estrogen 4 hours, (8) estrogen plus atropine 4 hours, (9) estrogen plus progesterone 4 hours, (10) atropine 4 hours, (11) estrogen 48 hours, (12) estrogen plus progesterone 48 hours, (13) castrate rabbit uterus estrogen treated, (14) castrate rabbit uterus, untreated.

overdose of nembutal, their tissues were examined immediately and then fixed in formalin for subsequent re-examination.

Upon examination, it was seen that the uteri of animals treated with estrogen were stained an intense blue, while those of the castrate controls had only a slight bluish discoloration. Figures 1 and 11 illustrate representative uteri from both groups. Since the hyperemic effects of the estradiol benzoate might significantly influence the color of the uterus by increasing the amount of blood and hence of the dye in the tissue, it was necessary to ascertain the distribution of the dye within the uterus. Sections of the uteri of both groups were cut at 40μ and faintly counterstained by a very dilute solution of eosin in xylol. The amount of trypan blue in the vascular channels of the

castrate and of the estrogen treated uteri was approximately the same. The control castrate uteri, when examined histologically, had no trypan blue within the uterine tissue, while the estrogen treated uteri contained significant amounts. This blue seemed to be concentrated in the endometrial stroma, little or none being seen in the lining epithelium, the glands or in the muscularis. This finding indicates that the increased amount of trypan blue seen macroscopically in estrogen stimulated uteri, is due mainly to the effect of estrogen on capillary permeability.

In order to check the possibility that the trypan blue intake by the estrogen stimulated uterus (excluding capillaries) did not depend on changes in blood volume or slight differences in the amount of dye injected from animal to animal, control castrated rats were injected with 1.5 to 2 times the dosage of trypan blue. Representative uteri are illustrated in figures 2 and 3. It will be seen that the administration of more dye definitely increases the color of the castrate uterus. The castrates which had received 1.5 and 2 times the amount of trypan blue were stained correspondingly more deeply than castrates receiving the original amount of the dye. Nevertheless, if one compares the trypan blue content of estrogen stimulated uteri with that of the castrates receiving the increased dosage of the dye, it is apparent that the stimulated uteri contained more trypan blue than the uteri from the rats which had received 1.5 times the original dosage, and that they contained approximately as much as those of the castrates which had received twice the dosage. On histological examination, it was apparent that the increase of color in the castrates receiving larger amounts of dye appeared to be due to an increased amount of dye in the vascular channels, little or no trypan blue being found within the uterine tissue. In similar fashion, it has been possible to produce deeply stained estrogen stimulated uteri with lower concentrations of the dye than were used in the above experiments.

These results demonstrate that the trypan blue intake by uterine tissue, exclusive of the vascular channels, is but slightly dependent upon the trypan blue concentration of the blood in the range of the dosage employed. Slight differences in the blood dye concentration, due to blood volume changes, or slight variations in the amount of dye administered, are incapable of explaining our results.

In order to establish the fact that this reaction was characteristic for more than one species of animal, the same experiments were performed using castrated rabbits as test animals. Six castrated rabbits received a single injection of 2,000 I.U. of estradiol benzoate subcutaneously and 48 hours later they were injected intravenously with 2.5 cc. per kg. body weight of a 1% trypan blue solution in saline. One hour after the injection of the dye, the animals were killed and their uteri examined. Comparison of these uteri with the uteri of uninjected castrates treated in a similar fashion, showed that the estrogen stimulated rabbit uteri, just as in the rat, were stained intensely. The uteri of the control castrates had accumulated none of the dye. These tissues are compared in figures 13 & 14. These experiments demonstrate that estrogen stimulated uteri of rats and rabbits show a markedly increased capillary permeability to trypan blue.

Time interval of reaction. Having established the fact that estradiol benzoate causes an increased capillary permeability, it was of interest to find the time interval between hormone injection and the appearance of trypan blue accumulation. In order to establish the primacy of increased capillary permeability over the fluid accumulation which estrogen produces, it is necessary that the former occur before or at the same time as the latter. To this end, castrated rats were injected with estradiol benzoate intramuscularly. These animals were killed at intervals of 1, 2, 3, 4 and 24 hours after the estradiol benzoate injection. One-half hour before each of these animals was killed, trypan blue was injected intravenously. Table 1 illustrates these findings.

TABLE 1

No. rats	Hr. after single dose of 100 I.U. estradiol benzoate	Results
6	0	Little or no trypan blue
3	1	1+(?) accumulation
3	2	1+ accumulation
4	3	2+ accumulation
4	4	3+ accumulation
5	24	3+ accumulation

Figures 1 to 12 illustrate the macroscopic picture of representative uteri. It will be seen that there are definite differences in the trypan blue content between castrate and estrogen injected uteri. These differences are apparent within 2 hours after the intramuscular administration of estrogen, and are maximal at 4 hours. Sections of these uteri show considerable uniformity in the amount of intravascular blue. Astwood (1) has shown in the rat that uterine water accumulation begins at 2 hours and is very pronounced 6 hours after the injection of estrogen. The similarity in the time intervals between fluid accumulation and the increased capillary permeability demonstrated in our studies, suggests that the water accumulation may be due in part to the increased capillary permeability. Time interval experiments were also performed with rabbits. Table 2 illustrates these findings.

TABLE 2

No. of rabbits	Hr. after inj. of 2000 I.U. estradiol benzoate	Dye accumulation
3	1	0
3	2	0
4	4	+
6	6	+

It will be seen that the reaction in rabbits is also rapid and may occur as soon as 4 hours after administration of estrogen. The differences in the results between rats and rabbits is not large, and may be due to differences in the technic employed.

Effect of atropine. It has been shown by Reynolds (5) that estrogen has a cholinergic action on the uterus of the rabbit, but not on the uterus of the rat and cat. It has also been shown that the mouse resembles the rabbit, since in the former large doses of atropine inhibit the hyperemic effects produced by estrogen 24 and 48 hours after administration (6). We were interested, therefore, in determining whether or not the effect of estradiol benzoate on the permeability of the uterine capillaries was mediated by way of acetylcholine.

In these experiments, both rats and rabbits were used. Four castrated rabbits were injected with 2000 I.U. of estradiol benzoate intramuscularly, and were thereafter given 5 mg. atropine sulphate per kg. body weight subcutaneously at 30-minute intervals for 5½ hours. The trypan blue solution (2.5 cc. of a 1% trypan blue in saline per kg. body weight) was injected intravenously and the uteri were examined 30 minutes later. Control rabbits which received estrogen without atropine were treated in exactly the same fashion. Upon examination, both groups of uteri were markedly stained and no appreciable differences were seen between the atropinized and control uteri.

Ten castrated rats were divided into 2 groups. All rats received an initial injection of 100 I.U. of estradiol benzoate intramuscularly. Counting the estrogen injections as zero time, all rats were injected subcutaneously with 10 mg. of atropine sulphate at 0, 1, 2 and 3 hours. Two and one-half hours after the initial injection, one group was in-

jected intravenously through the jugular vein with trypan blue and killed ½ hour later. The second group was injected at 3½ hours with trypan blue, and killed 4 hours after the initial injection. The uteri from these animals were compared with those of controls which had received estrogen only for similar periods of time, and with those of castrates which had received atropine sulphate without estradiol benzoate.

Macroscopically, the uteri of the animals which had received both atropine and estrogen had been stained blue to approximately the same extent as those which had received only estrogen. Animals which had been given only atropine showed little or no differences in the uteri from those of castrates. Figures 6 to 10 illustrate the macroscopic appearance of representative uteri from various groups. Microscopic examination confirmed the macroscopic findings. However, on cutting sections at 8μ, and staining with hematoxylin and eosin, certain differences were perceived between the uteri of animals receiving estrogen and those receiving estrogen and atropine. The loosening of the endometrial stroma, induced by estrogen, seemed to be inhibited slightly but definitely by the simultaneous and continuous administration of atropine.

The results in rabbits and rats indicate that atropine cannot inhibit the capillary permeability response of the uterus to estradiol benzoate. Although we cannot be sure that full effective atropinization of the uterus was produced, the doses of atropine employed were both large and continuous. The finding that these amounts of atropine, insufficient to affect capillary permeability, were still able to inhibit fluid accumulation as judged histologically further suggests that effective concentrations were employed.

Effect of progesterone. The simultaneous injection of 5 and 10 mg. of crystalline progesterone had little or no effect on the capillary response induced with 100 I.U. or 2,000 I.U. estradiol benzoate in rats and rabbits respectively at 4 and 48 hours after the initial injection (fig. 9 and 12).

DISCUSSION

Our results indicate that estradiol benzoate increases the permeability of the uterine capillaries to trypan blue. It is further shown, that the onset of the reaction is rapid and parallels in time, the fluid accumulation produced by the female sex hormone. This suggests that the uterine fluid accumulation is, in part, the result of estrogen action on the permeability of the capillary membranes.

Experiments on rabbits and rats, wherein large continuous doses of atropine fail to inhibit the trypan blue intake induced by estrogen, indicate that this effect on capillary permeability is independent of the action of estrogen in liberating acetylcholine. The significance of these atropine experiments lies in the demonstration that atropine was able to inhibit, in part, the fluid accumulation induced by estradiol benzoate, as judged by histologic study. This result may mean that more than one factor is involved in the production of uterine edema. If the capillary permeability effect of estrogen is assumed to be one of the factors involved, then the existence of another operative factor which is sensitive to atropine must be postulated. This work confirms and extends previous studies in the mouse uterus (6), wherein it was shown that large doses of atropine incompletely inhibits the effect of estrogen on fluid accumulation. It appears that two factors at least are involved in the reaction; one of which is atropine sensitive, the other, atropine insensitive. The atropine insensitive factor appears to be related to the effect of estrogen on capillary permeability. The atropine sensitivity of the other factor, strongly suggests that acetylcholine is involved in the operation of that mechanism. In considering the nature of this atropine sensitive factor the fact that atropine inhibits the uterine hyperemic effects of estrogen in mice (6) seems to be important in that it indicates that acetylcholine may be involved in the

production of estrous hyperemia in the mouse. If the estrous hyperemia is associated with increased capillary filtration pressures, then it is possible to relate atropine sensitivity to a factor responsible for producing edema. According to this view, therefore, estrogen promotes fluid accumulation in the uterus not only by producing an increase in the permeability of the uterine capillaries, but perhaps also by liberating acetylcholine which increases the capillary filtration pressure.

It is recognized that there are two objections to this theory, which cannot at this time be answered. First, the assumption that estradiol benzoate is cholinergic in the rat does not agree with the work of Reynolds (5) who found no increase in the content of free acetylcholine in the rat uterus following estrogen administration, although the rabbit uterus does respond in such a fashion. However, our experiments wherein atropine produced definite inhibition of the uterine edema of estrus, can be explained by assuming that estradiol benzoate is cholinergic in the uteri of rats as well as of rabbits. The quantitative aspects of acetylcholine liberation by estrogen in rat uteri may be such that amounts sufficient to influence fluid accumulation are insufficient to produce a measurable increase in the free acetylcholine content. Second, the assumption that the uterine capillary pressure is increased by estrogen through acetylcholine seems to be incorrect in view of the demonstration by Holden (7) that atropine does not inhibit the hyperemia and vasodilatation produced by estrogen. Too, acetylcholine does not produce these vascular reactions in the uteri of infantile rats. Investigations of this phase of the work are now in progress.

The possibility that the uterine edema of estrus is due to changes in the normal osmotic relations between plasma and tissue fluid can be partially evaluated. If estrogen produced a decrease in the osmotic pressure of plasma without affecting the osmotic pressure of tissue fluid, then the edema which is produced should be generalized, and not localized. Since local uterine edema only is produced with small amounts of estrogen, it is unlikely that the uterine effects of estrogen are mediated by way of this mechanism. If, however, estrogen selectively produces an increase in the osmotic pressure of uterine tissue fluid, then this reaction may be responsible for producing localized edema. Our results throw no light on this possible mechanism. The possibility that estrogen may act by obstructing the lymph channels, or by interference in lymph drainage has not been quantitatively evaluated in this work. However, if such a factor were operative, lymph stasis and lymphatic dilatation should be observed in sections of estrous uteri. The absence of these findings must be interpreted as evidence that lymphatic obstruction plays no significant part in the uterine fluid accumulation produced by estrogen.

From this discussion, it appears that at least two activities of estrogen account for its characteristic uterine edema effects: *a*), the increased capillary permeability effect of estrogen; and *b*), some atropine sensitive factor which has been suggested to operate through an increase in capillary filtration pressure. The possibility that estrogen causes edema by increase in the osmotic pressure of uterine fluid (for example, by salt retention), has not been accounted for. However, edema production as the result of lowering of plasma osmotic pressure, or by lymphatic obstruction can be excluded.

The effect described for estrogen on the uterine capillaries appears to be relatively specific. Preliminary experiments suggest that estradiol benzoate produces increased capillary permeability in the vagina as well as in uterus, but not in other tissues. These observations will form the basis for a subsequent report.

SUMMARY

Estradiol benzoate produces a rapid increase in the permeability of the uterine capillaries to intravenously administered trypan blue in both rats and rabbits. Large

doses of atropine and progesterone are without significant effect on this reaction. The relationship of this finding to the fluid accumulation produced by estrogen in the uterus and in the generalized tissues is discussed.

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UTERINE DISTENTION AND LACTATION[1]

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SELYE,[2] and Selye, Collip and Thomson[3] published a series of experiments which were interpreted as showing that distention of the uterus would inhibit lactation. Rats which had their uteri distended with paraffin on the day following normal parturition or at the time of a Caesarian section failed to lactate, and the mammary glands showed signs of involution as early as the fourth day after the termination of pregnancy even in spite of active suckling. It was inferred that the distended uterus inhibited the release of the lactogenic hormone from the pituitary. If these claims could be substantiated this type of animal should be excellent for assaying the lactogenic hormone, prolactin. The present report cites our experience which fails to substantiate the original claims.

The technic used in this study closely approximated that described by Selye. Rats were selected for operation during the first 24 hours after a normal delivery. The uterus was exposed through an abdominal incision while the rat was under an ether anesthesia. Paraffin (melting point 42° C.) was injected into the uterus from a 10 cc. hypodermic syringe so that the volume injected could be measured. The number of fetuses that had been in each uterine horn was easily determined by noting the nodules at the implantation sites. A volume of paraffin exceeding 2 cc. per fetal implantation site frequently ruptured the uterus or forced the paraffin into the uterine veins especially if the paraffin was too warm. The partly solidified paraffin was injected so slowly that it was frequently necessary to apply heat to the 20-gauge needle to keep the paraffin flowing. The needle was inserted through the cervical portion of the uterine wall and then a silk ligature was tied around this portion of the uterus to prevent explusion of the paraffin. Seven rats were subjected to Caesarian section on the day before full term delivery was expected. In order to achieve a maximum degree of distention without the danger of paraffin emboli the uterine horns were filled with soft paraffin pellets which roughly approximated the size and volume of the fetuses just removed. A much greater volume of paraffin was introduced into these uteri than was possible in the postpartum rats.

The mother rat was weighed immediately after the operation and the weight of the litter or the foster young was also noted. Daily weights of the mother and litter were recorded for the next 2 or 3 weeks. Notes were made several times during the immediate post-operative period as to whether the mother rat was allowing the young to suckle or whether she was staying away from the nest.

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[1] Read before the twenty-fifth annual meeting of the Association for the Study of Internal Secretions on May 2, 1941 in Atlantic City, N.J.

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EXPERIMENTAL

Of 21 mother rats subjected to various degrees of uterine distention (2 cc. to 20 cc. per uterine horn) 5 died immediately from paraffin emboli; 2 were moribund and were killed after 24 hours and 48 hours respectively. The remaining 14 rats demonstrated their ability to rear litters of 4 to 8 rats up to weaning age.

Figure 1 illustrates the initial weight loss of the mother rat with a paraffin distended uterus, and of her litter as compared with an untreated normal control. Allowing for the 3-day postoperative delay in litter weight gain, the subsequent gain parallels that of the normal mother. When 20 cc. of paraffin was injected intraperitoneally, the mother and the litter show a weight loss comparable to that for the intrauterine injection. Further experiments would reveal whether abdominal distention and discomfort would be equally as effective as uterine distention in inhibiting lactation.

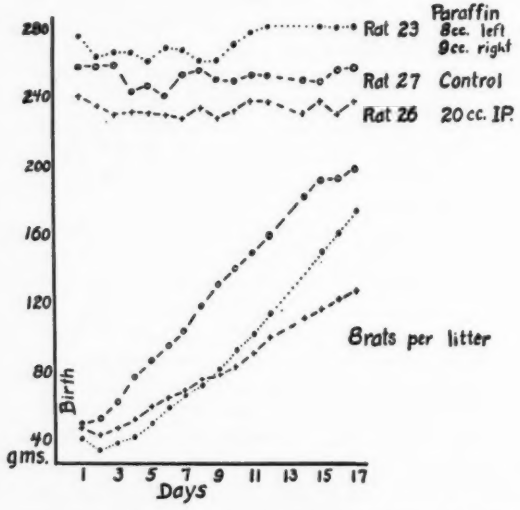

Fig. 1. Daily weights of 3 mother rats and their respective litters. *Rat* 23 had a total of 17 cc. of paraffin in her uterine horns. *Rat* 26 was an operated control with 20 cc. of paraffin in the peritoneal cavity but none in the uterus. *Rat* 27 was a normal unoperated control.

Of the 7 rats subjected to Caesarian section, 4 died within 48 hours and 2 of the other 3 survived 9 days and the other one 13 days. One of these had sufficient milk to nourish a litter of 6.

There was an apparent failure of lactation in several rats as illustrated in figure 2, by the death of the original litter after about 48 hours. This occurred in both the postpartum and the Caesarian rats. However, when a vigorous foster litter was put with the mother rat these foster young succeeded in getting enough milk to grow normally. Close observation revealed that the operative procedure causes so much abdominal discomfort that the mother usually does not give the young any attention for the first 24 hours. She has very noticeable abdominal cramps and is very apathetic. During this time the young may be scattered over the floor of the cage or left in the nest, cold and starved. If this period of starvation exceeds 36 hours the young become too weak to suckle even if placed with a normal lactating foster mother. When the

young were allowed to nurse a foster mother until placed again with their own mother after she has been allowed a 24-hour postoperative recovery period, or when well nourished vigorous foster young were placed with the operated mother after this recovery period this mother consistently demonstrated her ability to lactate even though the uterus was distended with paraffin.

Unfortunately Selye did not record the volume of paraffin injected but his judgment of a 'distended' uterus was based on experiences similar to ours with ruptured uteri and paraffin emboli (personal communication). From our experience (protocols

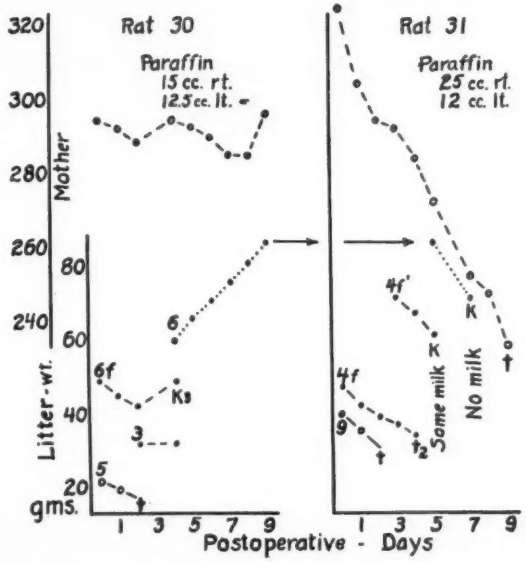

Fig. 2. Daily weights of 2 mother rats and litters following caesarian section. The original litters of 5 and 9 rats died within 48 hours. On the 4th day, 3 of 6 foster young with rat 30 were killed (K_3) and then the remaining 6 made fairly normal daily weight gains. *Rat 31* continued to lose weight, 90 gm. in 9 days, but she did have some breast secretion as indicated by the presence of milk in the stomachs of 4 foster young killed on the 5th day. The litter of 6 that had gained with *rat 30* were unable to get any nourishment from *rat 31* after the 5th day.

in table 1) it appears that 10 cc. per horn is the maximum possible distention except in Caesarian sectioned rats. Since this distention did not inhibit lactation it would seem that the experimental findings of Selye may have been misinterpreted. It is common knowledge that rats and mice will lactate during pregnancy and when the pregnant uterus is greatly distended. The experimental procedure is so severe a treatment of the mother that the young are neglected during a very critical period of adjustment. This neglect of the newborn may be sufficient to leave them too weak to nurse and brings an additional factor into the experiment which can be avoided by the use of well nourished foster young. Lactation will not occur unless the nipples of the mother rat are actively suckled and this stimulus is supplied by the foster litters.

Lactation is also dependent upon the nutritional state of the mother. Any traumatic insult sufficient to cause a prolonged anorexia and loss of weight will inhibit lactation. The operative procedure in this experiment caused a weight loss of 10 to

20 gm. in the first 2 postoperative days but after this time the maternal weight stabilized or tended to show a progressive gain during the rest of the lactation period.

TABLE 1

Rat no.	Paraffin volume		No. in litter	Litter weight at		
	Right horn cc.	Left horn cc.		10 days gm.	15 days gm.	20–21 days gm.
1	2	2	5	79	119	148
2	2	2	5	78	112	126
3	Sham operation		5	67	100	127
7	5	4	5	80	110	139
9	5	5	5	68	103	129[1]
10	10	10	Survived only 48 hr., 44 gm. loss of wt.			
11	10	10	Moribund at 24 hr.			
12	10		Paraffin embolus			
13	10	—	Paraffin embolus			
14	10	10	Paraffin embolus			
21	10	9	7	80	122	167
22	7	5	6	105	135	149[2]
23	9	8	8	96	154	201
26	20 cc.[3]		8	87	120	155
27	control		8	143	195	206[2]
30	15	12	6 normal gain 4th to 9th day (fig. 2)			
31	25	10	starved 4 litters in 9 days (fig. 2)			

[1] Killed on the 18th day.
[2] Killed on the 19th day.
[3] Paraffin injected intraperitoneally.

It was very evident that the peritoneal injury during the Caesarian section produced such a profound shock that little or no anesthesia was needed while the paraffin was placed in the uterus and the incision was closed.

SUMMARY

Distention of the uterus does not prevent lactation in the rat. The operative technic employed is so hard on the mother that she neglects the newborn young until they do not have enough strength to suckle and die of starvation, but if she is given a vigorous foster litter it will make normal weight gains.

EFFECT OF TESTOSTERONE PROPIONATE UPON THE MITOTIC ACTIVITY OF THE ADRENALS IN THE INTACT IMMATURE FEMALE RAT

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PREVIOUS STUDIES in this laboratory (1, 2) have indicated that testosterone propionate acts directly upon the vagina and uterus of the immature female rat, and stimulates the ovary indirectly by way of the hypophysis (1) even in the absence of the adrenals (2). Moreover, it was shown that testosterone was capable of stimulating the thyroid and parathyroid glands of the same type of animal (3). This stimulation was manifested by increased mitotic activity and by histological evidence of functional activity. The mechanism by which this occurs is probably an indirect one via the pituitary gland. It is the purpose of this communication to record the effect of testosterone upon the mitotic activity of the adrenals in the intact immature female rat. Colchicine was used in addition to testosterone because of its ability to temporarily inhibit or prolong the mitotic cycle in early metaphase. In this way the number of mitoses occurring during any given short period of time may be estimated.

EXPERIMENTAL METHODS

Group I. Twenty immature female rats of the Wistar strain, weighing between 40 and 48 gm., and 4½ weeks of age were divided into 2 groups. Five mg. of testosterone propionate[1] in 0.2 cc. of sesame oil were given in one dose to each of the first group of 11 rats and 0.2 cc. of sesame oil only was given to the second control group of 9 rats.

Sub-group A (Testosterone treated). Twelve hours after testosterone was given, colchicine (0.05 mg. per 50 gm. of body weight) was given subcutaneously to one animal. The animal was killed 12 hours later. Thereafter one animal was killed every 24 hours, a single injection of colchicine having been given 12 hours previously.

Sub-group B (Controls). Three animals each were killed at 72, 144 and 288 hours respectively after the onset of the experiment. Two of each group of 3 were given colchicine 12 hours before.

Because of the findings, a second group of animals was studied more intensively at 48 and 96 hours after the administration of the hormone. This was done primarily to determine the consistency of the stimulation and secondly to ascertain the relative effect of colchicine at various intervals after its injection.

Group II. Twenty-nine immature female rats of a laboratory strain, 4½ weeks of age and weighing between 40 and 45 gm. were divided and treated as follows.

A) Testosterone and colchicine.

1) To 6 rats, a single dose of 5.0 mg. of testosterone propionate in 0.2 cc. of sesame oil was given. Forty-eight hours thereafter colchicine was administered and a rat was killed at 2, 4, 6, 8, 10, and 12 hours thereafter.

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[1] Testosterone propionate, in the forms of Perandren and Oreton, was supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J., through the courtesy of Dr. Ernst Oppenheimer, and by the Schering Corporation, Bloomfield, N. J., through the courtesy of Dr. Gregory Stragnell and Dr. Max Gilbert.

2) To 6 rats testosterone propionate was given as in the previous group, but 96 hours thereafter colchicine was given and the animals were killed at the same time intervals as above.

B) Testosterone only.

1) Two animals were killed at 52 and 56 hours respectively after the administration of 5.0 mg. of testosterone propionate.

2) Three animals were killed at 100, 104 and 108 hours respectively after testosterone propionate.

C) Colchicine only.

1) Five rats were given colchicine at the same time as *Group II*, A 1 and were killed at 2, 6, 8, 10 and 12 hours thereafter.

2) Five rats were given colchicine at the same time as *Group II*, A 2 and were accordingly killed at 2, 4, 6, 8 and 10 hours.

After killing the animal the adrenals were fixed in Bouin's solution, imbedded in paraffin and representative sections were stained with phloxine methylene blue. Mitoses were counted using the method of Brues and Marble (4). A minimum of 4000 cells were counted in each gland and at least one entire section per gland was examined.

RESULTS

Tables 1 and 2 present the essential findings in these experiments.

TABLE 1. TIME EFFECT OF TESTOSTERONE ON MITOSES OF ADRENAL GLAND. GROUP I[1]

Rat no.	Age when sacrificed, days	Hours after testosterone	Mitoses per 10,000 cells	Remarks
		Sub-group A (Testosterone treated)		
114	32	24	37	Colchicine
103	33	48	117	Colchicine
115	34	72	56	Colchicine
105	35	96	76	Colchicine
111	36	120	21	Colchicine
101	37	144	36	Colchicine
102	38	168	16	Colchicine
109	40	216	22	Colchicine
104	41	240	10	Colchicine
107	42	264	8	Colchicine
113	43	288	6	Colchicine
		Sub-group B (Controls)		
116	35	—	5	No colchicine
117	35	—	8	Colchicine
110	34	—	36	Colchicine-cyst pituitary
108	38	—	7	Colchicine
118	39	—	15	Colchicine
119	39	—	5	No colchicine
112	43	—	6	Colchicine
120	44	—	8	Colchicine
121	44	—	6	No colchicine

[1] The mitotic activity of the thyroid and parathyroid glands in this same series has been published previously (3). Comparisons between the glands may be made by referring to the number of the animal.

DISCUSSION

It can be seen from table 1 that testosterone propionate is capable of stimulating the adrenal glands of the intact immature female rat. This increase in activity is most marked in the first 96 hours and gradually decreases thereafter until after 216 hours the values are the same as those for the controls.

The results as shown in table 2 not only amplify but verify the consistency of the stimulating effect of the hormone on the adrenal cortex. The increase in mitotic activity, particularly within the first 96 hours, closely parallels the changes seen in the thy-

TABLE 2. GROUP II

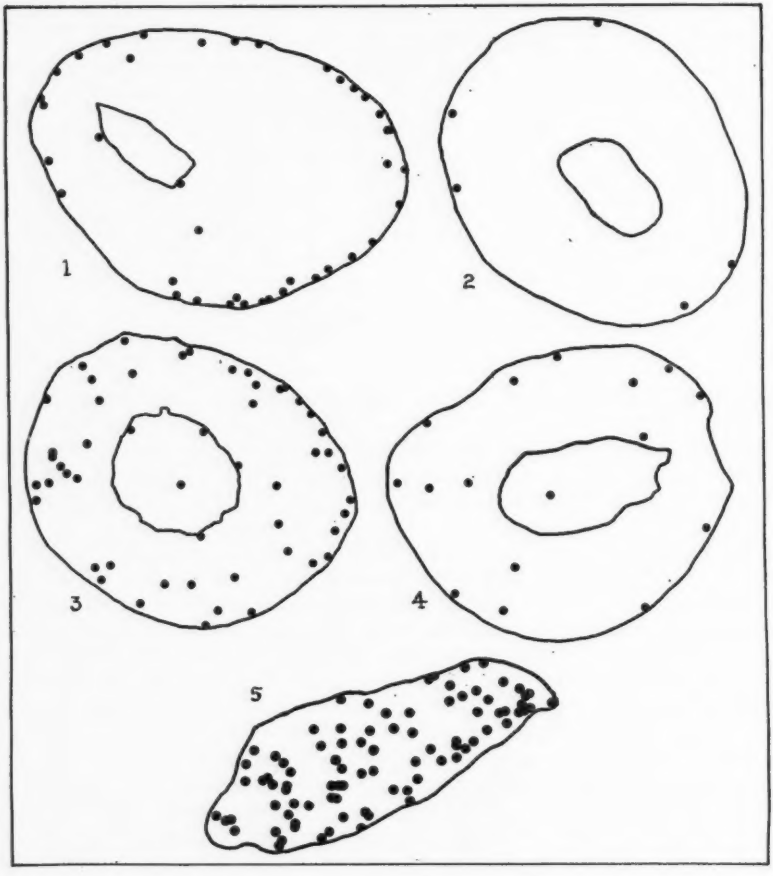
Hours after colchicine	Colchicine only, mitoses per 10,000 cells	Testosterone and colchicine killed at 48 hours, mitoses per 10,000 cells	Testosterone and colchicine, killed at 96 hours, mitoses per 10,000 cells
0	—	19	4
0	—	12	6
0	—	—	7
2	31	65	49
	24	—	—
4	40	81	86
6	42	95	57
	36	—	—
8	28	104	75
	26	—	—
10	23	50	68
	38	—	—
12	30	144	52

roid and parathyroid glands and ovaries under the same treatment and during the same period of time. The maximum effect in the adrenal occurs at 48 hours, which is somewhat earlier than the other glands which show a peak of activity at 96 hours. This may be taken, perhaps, to indicate that the phenomenon is not a general stimulus to tissue proliferation, but a stimulation of the individual gland to a process which involves growth.

Of particular interest is the distribution of mitoses in the gland. In *Group I* the mitoses were almost strictly confined to the capsule, the zona glomerulosa and the outermost layers of the zona fasciculata. Mitoses were occasionally seen elsewhere in the zona fasciculata and the zona reticulosa. None were encountered in the medulla (fig. 1, 2). In *Group II*, as in *Group I*, the greatest number of mitoses appeared in the capsule, zona glomerulosa and outer zona fasciculata. Of note, however, is the fact that mitoses were relatively frequent in the deeper layers of the zona fasciculata, the zona reticularis and the medulla, as compared to *Group I* (fig. 3). This activity amounted to 10 to 20% of that seen in the outer zona and was present to some extent in the untreated animal of *Group II* (fig. 4). This, as well as the variation in mitotic activity in the two groups, particularly in the controls, may be attributed to the difference in the strain of rats used, since all of the other conditions were the same. The fact that mitoses were present in the medulla of one strain and not in the other contributes to this idea. It emphasizes the necessity of using single strains for any particular experiment and for adequate controls if different strains are used in the same study. The apparent presence of mitoses in cells of the medulla makes it appear that this area is also stimulated. Our data are too few for further speculation. One control animal (No. 110) deserves comment since the number of mitoses was comparable to those of the testosterone stimulated glands. This animal also had stimulation of the ovary, thyroid and parathyroid glands. Autopsy revealed a cyst of the pituitary gland, which may indicate a possible alteration of function of the gland.

The localization of these mitoses is of importance for three reasons. First, it suggests that the cortical cells arise in the periphery of the gland and move inward as they mature. This conforms to the commonly held view which has been recently substan-

tiated and well summarized by Bennett (5). He states that "the cells of the adrenal cortex are continually migrating from their place of formation in the capsular or subcapsular region toward the medulla, near which they degenerate." Secondly, Bennett (5) has demonstrated that chemical substances in the cortex of the adrenal gland of the cat with reactions similar to those of biologically active cortical sterones are usually confined exclusively to the outer layer of cells in the zona fasciculata. These findings,



DIAGRAMS OF POSITION OF OBSERVED MITOSES IN CROSS SECTIONS OF REPRESENTATIVE GLANDS. (Black dots indicate mitoses.) Fig. 1. Adrenal gland of rat treated with testosterone, Group I. Fig. 2. Adrenal gland of control rat, Group I. Fig. 3. Adrenal gland of rat treated with testosterone, Group II. Fig. 4. Adrenal gland of control rat, Group II. Fig. 5. Thyroid gland of rat treated with testosterone.

together with an increase in mitoses in the outer zones of the cortex of the adrenal gland, suggest a significant correlation between physiological and histological activity. Thirdly, in contrast to the localized areas of the mitoses in the adrenal gland, the mitoses in the thyroid and parathyroid glands were uniformly distributed throughout (fig. 5). This suggests that the latter glands are uniform in their functional activity.

The action of colchicine is amply demonstrated in this study. This is best shown by the differences in demonstrable mitotic activity as seen in table 2, between the animals treated by colchicine alone, testosterone alone and by a combination of the two. It is of interest that the animals treated by colchicine only had higher mitotic counts than those treated by testosterone alone. When both were used the mitoses were markedly increased over that when either were used alone. It is usually considered that the action of the drug is at its height between 6 to 12 hours after its administration. This appears to be the case in the animals killed 48 hours after testosterone was given, but it does not hold in the 96-hour period. The data, of course, are too few to draw any conclusions regarding the rate of mitotic accumulation following colchicine treatment.

The mechanism of the stimulation of the adrenals by testosterone is not entirely clear, although only several possibilities seem tenable: (*A*) testosterone stimulates the gland directly; (*B*) testosterone may stimulate the adrenal indirectly by way of the hypophysis.

The second of these theories seems more likely, especially since the changes parallel so closely those in the ovary which we have shown are dependent upon the anterior hypophysis. From this and our preceding studies, it is obvious that the action of testosterone is not confined to a single endocrine gland. This suggests, therefore, a general increased activity of the anterior pituitary, which in turn stimulates the other organs of internal secretion analogous to its effects at puberty. This view is fortified by the fact that the testosterone treated animals showed significant gains in size and weight over the untreated controls (6). Experiments in hypophysectomized animals and studies of other glands of internal secretion in these same animals are under way at present to clarify these points.

CONCLUSIONS

Testosterone propionate is capable of stimulating the adrenal glands of the intact immature female rat. This stimulation is manifested by an increase in mitotic activity which is most marked during the first 96 hours after its administration.

The location of the mitoses in the gland suggests that (*a*) the cortical cells arise in the periphery of the gland and move inward as they mature, and (*b*) there is a correlation between histological and physiological activity.

This increased mitotic activity parallels similar phenomena in the ovary, thyroid and parathyroid glands of the same animals. This, therefore, suggests a general stimulation of the anterior pituitary which in turn produces the observed changes in the peripheral organs of internal secretion.

We are indebted to Dr. Joseph C. Aub for many valuable suggestions and to Dr. George B. Wislocki for aid in the interpretation of histological material.

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FORTY-EIGHT-HOUR-RESPONSE OF THE IMMATURE MALE RAT TO ANDROGENS[1]

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THE AUTHORS HAVE REPORTED (1) that the prostate and seminal vesicle of the immature male rat show a definite weight increase 24 hours after the subcutaneous administration of testosterone propionate in oil. The rats used were 32 to 38 days old and weighed 65 to 85 gm. The mean responses of the seminal vesicles in the various groups increased consistently with the dosage. With the lowest dose used (0.00625 mg.) the increase in seminal vesicle weight above the normal control level was 10.59% and with the largest dose (0.5 mg.) the increase was 53.47%. The prostates showed a relatively smaller response than the vesicles and the response was also less consistent. While the mean seminal vesicle weights of the two highest dosage groups were significantly greater than the normal control mean, the differences between different dosage groups were not significant (in spite of the fact that there were 20–25 animals in each group). The individual responses within each group varied considerably.

The present communication deals with studies made to determine the cause of the large intra-group variability found in the previous study. The original technic was varied in an attempt to obtain a more consistent short time response to androgens in the rat. Some data (using the original technic) are presented which illustrate further the variability in response of the 32 to 38-day-old animals to androgenic substances.

The weights of the ventral prostates were obtained in all animals. Since these weights were more variable than the seminal vesicle weights, and since their responses were less consistent with the dosage they are not included in this paper.

Effect of larger doses of testosterone propionate over a 24-hour period. In our previous study 0.5 mg. testosterone propionate at a single injection was the highest dosage used. This dosage was repeated and also 1.0 mg. and 2.5 mg. were given to additional groups. The mean and individual seminal vesicle weights of these groups are shown in figure 1 along with the 0.5 mg. and control groups from our old data. While the 3 new groups of treated animals showed mean seminal vesicle weights which increased consistently with the dosage, the differences between the groups were very slight and all were below the mean obtained previously with 0.5 mg. testosterone propionate. In each group the range and variability were very great (see fig. 1).

Response to testosterone, 6 to 24 hour periods. Because of the slow absorption of esterified androgens, it was decided to test the free form, testosterone over 6 and 24-hour periods. As shown in figure 1 there was no demonstrable response to 0.4 mg. testosterone in 6 hours. In fact the mean seminal vesicle weight of this group was less

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than that of the controls, but not significantly so. In 24 hours there was a definite weight increase in the seminal vesicles. This response, however, was small for this fairly large dose of testosterone. In these groups, as in the previous ones, the intra-group variability was very great.

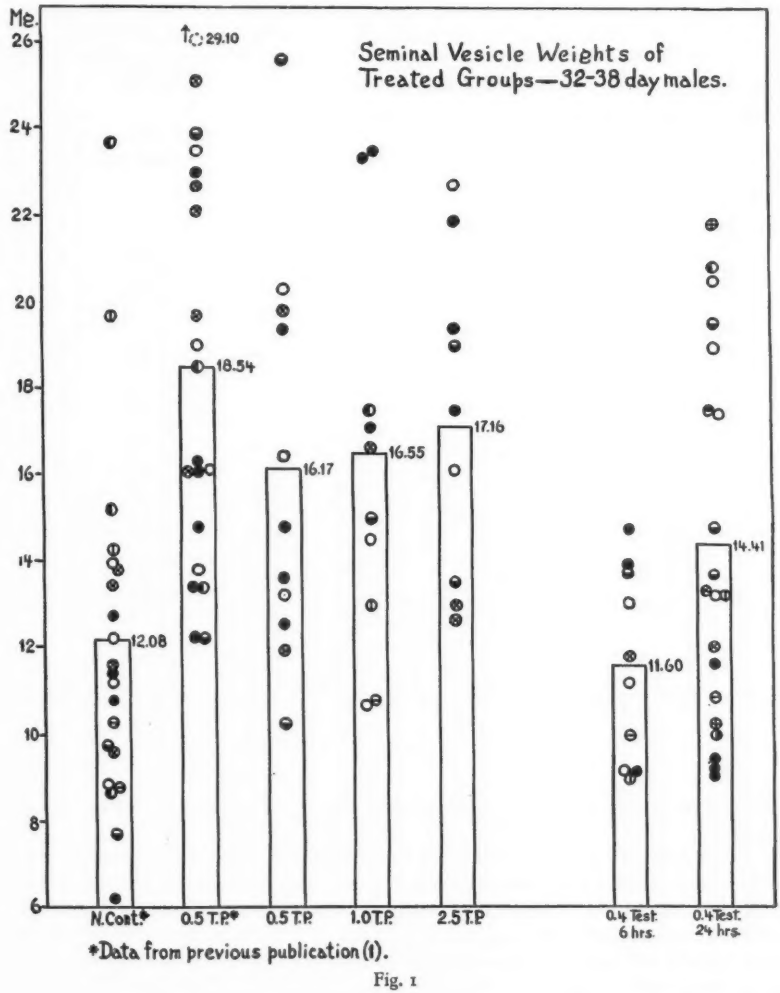

Fig. 1

Seminal vesicle variability in normal 33 to 38-day-old animals. In the original procedure and in the preceding groups the age and weight ranges of the animals were fairly extensive (32–38 days and 65–85 gm.). It was considered possible that both of these factors might have contributed to the large intra-group variability obtained. To test this possibility 10 animals of a strictly limited weight range (72–78 gm.) were killed for each day of an age range from 33 through 38 days. The seminal vesicle

weights of these animals are presented in figure 2a. The mean seminal vesicle weights were higher in the 3 older groups than in the 3 younger. The increase in mean seminal vesicle weight, however, was not very consistent with increasing age. For example, the mean of the 34-day group was larger than that of the 35-day group and the mean of the 36-day group slightly larger than that of the 37-day group. In addition, the variation in individual weights for animals of the same age was relatively

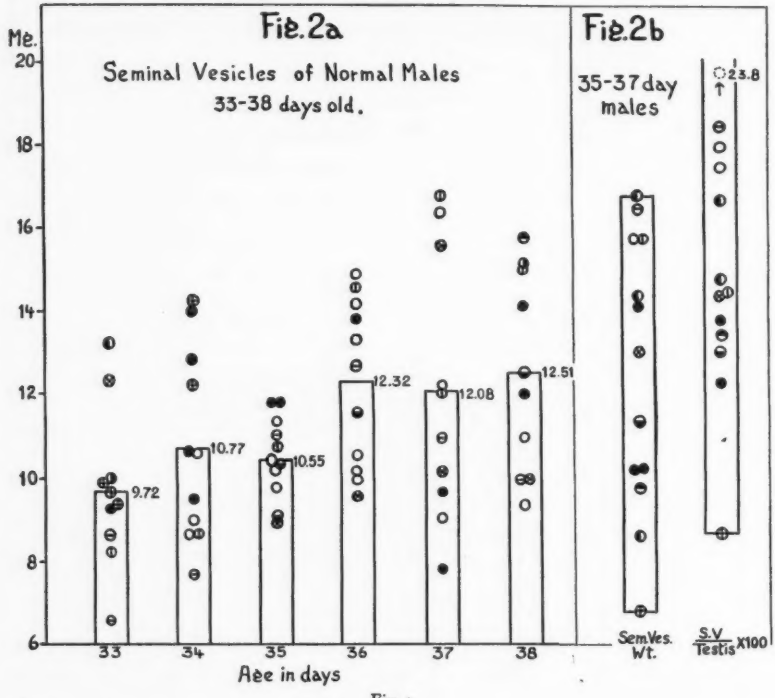

Fig. 2

tremendous. In each group the largest seminal vesicle was 75 to 115% larger than the smallest.

Correction for testicular weight. The inconsistent results of the last study, with both age and weight of the animals kept constant, prompted the thought that possibly the degree of seminal vesicle development in a group of animals of limited weight might be correlated with the degree of testicular development as reflected in testicular weight. If this were true, correction of seminal vesicle weights by means of testicular weights might be expected to give less variable results. Accordingly 13 animals of the same weight range (72–78 gm.), 35 to 37 days old were killed. The testes as well as the seminal vesicles of these animals were weighed. In figure 2b the uncorrected seminal vesicle weights are shown in the first column and the ratio of seminal vesicle to testis weight (S.V. Wt./Test. Wt. × 100) in the second column. The uncorrected seminal vesicle weights show a high degree of variability and, when corrected for testicular weights, do not show any less variability. It is possible that in a larger group of animals there might be a better correlation between testes and seminal

vesicle weights. However, it seems unlikely that such a correction would be of value in analyzing results in reasonably sized groups of treated animals of this age.

Seminal vesicle variability in younger animals. According to the growth curve of Moore and Price (2) there is relatively little growth of the rat seminal vesicles before the age of 32 days. It was therefore considered possible that intra-group variability might be minimized by using younger animals. The seminal vesicle weights of 14 males 22 to 24 days of age, weighing 26 to 36 gm., were obtained. They are presented in figure 3 (controls). The actual range in seminal vesicle weight was very much less than in the older animals. The relative range in these younger animals also was less, the largest vesicle was only 48% larger than the smallest, while in the older animals this value was 75 to 115%.

Forty-eight hour response in 20 to 22-day-old animals. In view of the lesser variability in seminal vesicle weight in the younger animals it was decided to determine

TABLE 1. 48-HOUR RESPONSE OF SEMINAL VESICLE WEIGHT TO ANDROGENS

Group No.	No. animals	Dose T.P.	Mean body wt.	Mean S.V. wt.	σ	C.R. of adjacent groups	C.R. of alternate groups
		mg.	gm.	mg.			
0	14	—	29.7	5.38	0.17		
						2.10	
1	10	0.005	29.8	6.20	0.35		4.99 (cont./0.01)
						2.30	
2	10	0.010	30.4	7.49	0.44		4.22 (0.005/0.025)
						2.42	
3	11	0.025	30.2	9.45	0.68		5.48 (0.01/0.05)
						2.09	
4	10	0.050	30.8	11.27	0.53		3.36 (0.025/0.1)
						1.47	
5	10	0.100	29.5	12.37	0.53		3.26 (0.05/0.25)
						1.57	
6	11	0.250	30.3	13.45	0.45		

$$\sigma = \text{Standard Deviation} = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

$$\text{C.R.} = \text{Critical ratio} = \frac{\text{obtained difference}}{\sqrt{(\sigma_{m_1})^2 + (\sigma_{m_2})^2}}$$

Critical ratios underlined = significant.

if their response to androgens would show less variability. Since in previous experiments no significant weight increment was obtained in a 6-hour period and since the maximal increment obtained in a 24-hour period was only 47 to 53%, a longer time interval (48 hours) was selected for trial in the hope that a larger weight increment would result.

The dose of testosterone propionate (0.5 mg.) which previously had produced the maximal weight increase was injected into 10 males, 20 to 22 days old. Forty-eight hours after treatment the weight of the seminal vesicles averaged 161% greater than that of the controls. Since this was a markedly greater response than any obtained previously it was decided to employ the same technic in other groups using smaller doses of testosterone propionate.

The animals used for this purpose were all 20 to 22 days old and weighed 25 to 32 gm. at the time of injection. The amount of solvent (sesame oil) was maintained constant, 0.1 cc. per animal. As close as possible to 48 hours after treatment (greatest

variation 15 minutes) the animals were killed. At autopsy their body weights varied from 26 to 36 gm. Under a dissecting microscope the coagulating glands were stripped from the seminal vesicles. The latter were then dissected free to their point

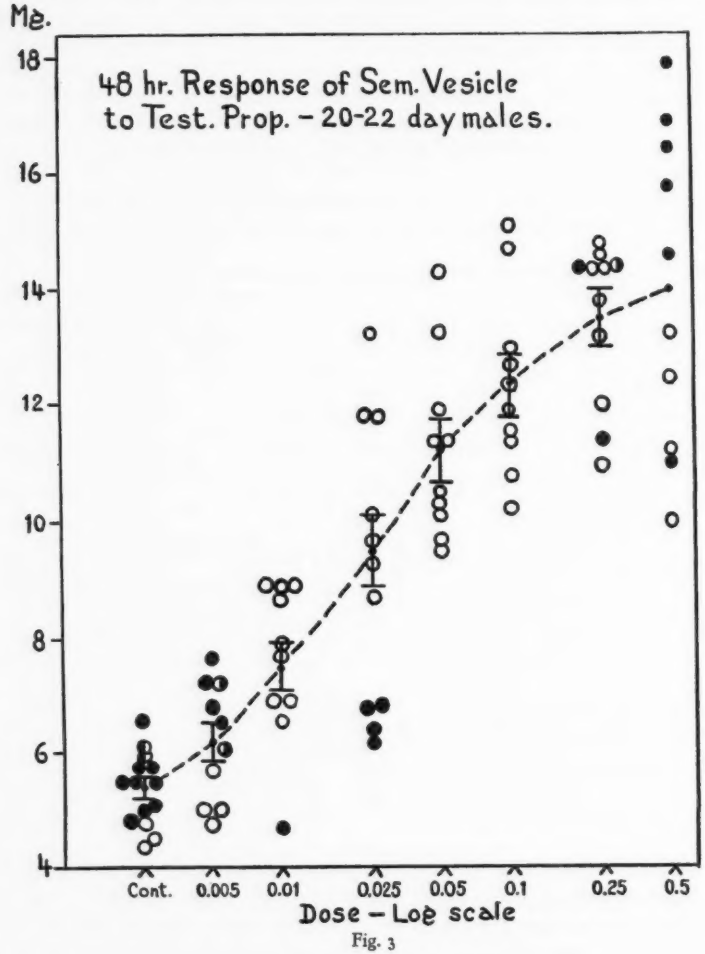

Fig. 3

of entrance into the ejaculatory duct, incised at this junction, removed and immediately weighed on a Roller-Smith milligram balance (sensitive to 0.02 mg.).

The doses of testosterone propionate used, the number of animals in each group, the results and the statistical analysis of the results are show in table 1. The seminal vesicle responses are shown in figure 3. The seminal vesicles show progressively greater responses as the dosage is increased. Although the differences between adjacent dosage groups are not significant, those between alternate groups are significant. For example, in table 1 the differences between *group 0* and *group 1* or between

groups 1 and 2 are not significant, but the difference between *groups* 0 and 2, or between *groups* 1 and 3 are significant.

DISCUSSION

The recent demonstrations of the inaccuracy of the chick method for androgen assays (3, 4, 5) stimulated publication of this data. It is quite possible that a less sensitive, but more accurate method of androgen assay could be based on the 48-hour response in the immature rat. Such a method would be more convenient than the current method using rats, because the immature animals do not have to be castrated and the time required for the test is very much shorter.

The dose-response curve presented in this paper could not be used for a standard dose response curve in an assay procedure since it was obtained with an esterified androgen. However, if a test similar to this one proves sufficiently sensitive to demonstrate the presence of amounts of androgen ordinarily found in biological assay material, a standard dose-response curve could be constructed very readily. Relative to such a procedure, it may be of interest that when older animals were used for a 24-hour period, the curve obtained by plotting response against the logarithm of the dose was a straight line, while the curve obtained with the younger animals for a 48-hour period was of the sigmoid type (fig. 3). It is not known whether or not this latter type of curve represents an artifact caused by the use of only 10 animals to the group. There is also a remote possibility that it reflects the influence of the administered androgen on the gonadotropic function of the hypophysis, thus secondarily affecting androgen production by the testes. This is assuming, of course, that such an effect would not be evident in the shorter (24-hour) period.

We have obtained the impression that part of the intra-group variability in seminal vesicle weight (particularly in treated animals) may be due to day-to-day variation, that is, the means of the weights in animals killed one day tend to show marked differences from the means in similar animals killed on other days. In the figures, the symbols for seminal vesicle weights in each group are similar for animals of that particular group killed on the same day. In figure 3 this day-to-day variation is particularly evident in some of the treated groups and also in the control group. This factor of day-to-day variation could be avoided in an assay procedure by administering the crystalline standard and the unknown to equal groups or sub-groups of animals on the same day.

The response which we have demonstrated is one of small magnitude, since differences amount to only a few milligrams in seminal vesicle weight. It is therefore necessary to use a meticulous technic in obtaining the seminal vesicle weights. This involves the use of a wide field binocular dissecting microscope to visualize the organs when freeing the seminal vesicles from the coagulating glands and in the removal of the vesicles themselves. To avoid drying and consequent weight loss the glands should be weighed immediately and as rapidly and accurately as possible. For this purpose we have found an ordinary balance inadequate since discrimination is insufficient and too much time is consumed. The Roller-Smith balance used in this study provides a rapid, convenient method combined with a degree of accuracy which appears to be adequate for the required purpose.

SUMMARY

The weight increase in the seminal vesicles 24 hours after administration of androgens is variable in 33 to 38-day-old rats weighing 65 to 85 gm. In an attempt to determine the cause of this variability it was found that untreated animals of the same

age and weight also showed marked variability in seminal vesicle weights. Correction for testicular weight did not decrease this variability.

The seminal vesicle weights of normal 22 to 24-day-old rats proved to be much less variable. Accordingly various doses of androgens were given to groups of 20 to 22-day old animals. When killed, 48 hours later, the mean seminal vesicle weights of the groups showed increases in weight consistent with the dose. Differences between alternate groups were statistically significant.

It is possible that an accurate assay procedure for androgens could be devised using this 48 hour response in 20 to 22-day-old rats.

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FEMALE MATING BEHAVIOR SHOWN BY MALE RATS AFTER ADMINISTRATION OF TESTOSTERONE PROPIONATE[1]

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STONE (1) and Beach (2) reported the execution of elements in the feminine copulatory pattern by male rats which were vigorous copulators in the normal masculine fashion. Various lines of evidence suggest the possibility that male rats possess neural mechanisms capable of mediating the mating behavior of the opposite sex, and that this overt behavior pattern may be manifested when the male, in a high state of sexual excitement, is repeatedly mounted and palpated by a vigorous copulator of his own sex.

Arousal of sexual behavior in prepubertally castrated male rats after administration of testosterone propionate (3), and restoration of mating in postpubertal castrates by the same method (4) indicates that sexual excitability adequate to the occurrence of normal mating is dependent upon androgenic substances. Beach (5) found that sexually inexperienced male rats injected with testosterone propionate prior to opportunity for intercourse with the receptive female displayed a tendency to mate with non-receptive female rats, young male rats and guinea pigs. The failure of most untreated control males to mate with incentive animals other than the estrous female of their own species indicated that testosterone propionate had raised the sexual excitability of the injected males above the normal level.

Utilizing this technic of producing 'hyperexcitable' males, and testing the response of such animals to the copulatory attempts of other members of their own sex, an attempt was made in the present study to determine the presence of the female mating pattern in the male rat.

EXPERIMENTAL METHODS[2]

Eight male rats 100 to 125 days of age were given 2 sex tests to determine the presence or absence of feminine copulatory responses prior to the injection of androgenic material. In each test 2 experimental males and one sexually-receptive female were placed in a circular cage 30 in. in diameter. By allowing each male to copulate with the female several times, but removing the female before ejaculation could occur, the experimenter attempted to create in both males a state of high sexual excitement. Under these conditions the males usually attempted to mount and palpate each other when the female was not available. Judging each male's excitability by the frequency and vigor of his attempts to copulate with the other male, test con-

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[2] Assistance in making daily injections and in the conduction of sex tests was rendered by Miss Priscilla Rasquin.

ditions were manipulated to increase excitability to a maximum in both individuals.

After 2 tests spaced two days apart each male was given a series of daily injections of 1.0 mg. of testosterone propionate.[3] On the 9th and 10th days of the experiment when each male had received 5 to 6 mg. of the hormone sex tests were repeated. Daily injections continued and tests were conducted on the 14th and 17th days after the administration of 10 to 13 mg. Two males which showed no tendency to mount other males and no indication of excessive sexual excitability were dropped from the experiment after they had received 13 mg. of testosterone propionate. The 6 animals remaining received an injection of 5 mg. on the 18th day, and a second injection of the same amount on the 22nd day. Each of these animals had received a total of 23 mg. at the conclusion of the injection program. On the 24th and 25th days the males were given a number of sex tests. Experimental technic was kept flexible to permit repeated testing and interchange of pairs when such procedure seemed likely to result in increased excitability of the males under observation.

RESULTS

Experimental results are summarized in table 1. Before the injection of testosterone propionate males showed a limited tendency to mount each other. After 5 to 6 mg.

TABLE 1. SUMMARY OF RESPONSES TO COPULATORY ATTEMPTS OF A SECOND MALE BEFORE AND AFTER INJECTION OF TESTOSTERONE PROPIONATE

	Rat	1	2	3	4	5	6	7	8
Before injection	No. tests	2	2	2	2	2	2	2	2
	Times mounted	9	0	0	2	2	1	3	2
	Reactions:								
	Fight	+			+	+	0	+	+
	Lordosis	0			0	0	0	0	0
	Hopping	0			0	0	0	0	0
	Ear movement	0			0	0	0	0	0
After receiving 5–6 mg.	No. tests	2	2	2	2	2	2	2	2
	Times mounted	35	0	3	12	16	2	3	4
	Reactions:								
	Fight	+		+	+	+	+	+	0
	Lordosis	0		0	0	0	0	0	0
	Hopping	0		0	0	0	0	0	0
	Ear movement	0		0	0	0	0	0	0
After receiving 10–13 mg.	No. tests	2	3	6	1	3	5	3	2
	Times mounted	30	8	23	2	30	0	29	6
	Reactions:								
	Fight	0	0	0	+	0		+	+
	Lordosis	0	7	7	0	0		0	0
	Hopping	0	6	1	0	1		0	0
	Ear movement	0	0	0	0	0		0	0
After receiving 23 mg.	No. tests		4	6		6	11	8	8
	Times mounted		18	31		70	10	76	72
	Reactions:								
	Fight		+	0		0	+	+	+
	Lordosis		10	9		16	0	0	3
	Hopping		3	0		0	0	8	0
	Ear movement		0	0		11	0	0	1

had been administered homosexual mounting increased. Seven of the 8 males were mounted 2 to 35 times but no feminine reactions occurred, and 6 of the 7 males fought when mounted.

[3] Crystalline testosterone propionate in sesame oil (5 mg. per cc.), in the form of Perandren, was supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

After receiving 10 to 13 mg. of the hormone 7 of the 8 males were mounted 2 to 30 times. Two males exhibited lordosis and hopping behavior typical of the sexually-receptive female and a third male displayed hopping without lordosis. Only 3 of the 7 males that were mounted showed fighting behavior. Two males were dropped from the experiment at this point.

After being injected with 23 mg. of testosterone propionate the remaining 6 males were mounted 10 to 76 times. One of the 6 cases showed lordosis and hopping behavior. Two displayed lordosis and ear vibrations characteristic of the estrous female. One exhibited lordosis only, one showed hopping, and the final animal displayed none of the elements of the female copulatory pattern.

Although the various reactions typical of the receptive female did not occur every time a male was mounted they were observed frequently enough to assure their presence in the male's behavior repertoire. *Rat 2* was mounted 26 times and exhibited lordosis in 17 instances. During the final series of tests *male 6* displayed lordosis 16 times and ear vibrations 11 times.

The feminine responses shown by injected males were difficult to elicit, sluggishly performed and quickly terminated. The behavior was comparable to that of a female in the early or late stages of estrus.

DISCUSSION

Present results would be more satisfactory if all males had been mounted as frequently in the preinjection tests as they were in tests after hormone administration. We might suspect that the feminine mating reactions depended upon the number of times a male was mounted rather than upon the effects of the hormone. In an attempt to check this possibility 4 non-injected males were given 3 sex tests with vigorous copulators and receptive females. The 4 males were mounted 88, 66, 48, and 22 times respectively. In no instance was any feminine behavior displayed by the mounted males.

Table 1 contains additional data indicating that feminine mating reactions are not purely a function of the frequency with which a male is mounted. *Male 1* failed to display any of the elements in the female pattern although he was mounted 67 times in the first 6 tests; whereas *male 2* was mounted only 8 times in the first 6 tests and showed lordosis in 7 of the contacts. *Rat 5* did not show lordosis in the first 7 tests although 13 mg. of hormone had been injected and the male was mounted 48 times. After receiving 22 mg. this animal showed lordosis the 27th time he was mounted.

It is important to note that males exhibiting feminine mating reactions displayed in the same tests vigorous masculine copulatory activity when placed with a receptive female. The behavior of such animals is bisexual rather than homosexual. There is no indication of a sex reversal, but rather an intensification of normal masculine mating behavior accompanied by occasional feminine responses.

Present results suggest several tentative conclusions. (*a*) Male rats possess neural mechanisms capable of mediating the mating reactions characteristic of the estrous female. (*b*) Endocrines do not organize this sexual behavior, but raise the excitability of nervous elements responsible for the organization. (*c*) Testosterone propionate increases the excitability of both male and female patterns in the male rat. (*d*) If the stimulus situation permits the occurrence of masculine copulatory behavior the testosterone treated male exhibits only the masculine mating reactions. (*e*) In the absence of opportunity for masculine copulatory reactions the highly excited male may exhibit the female response when mounted and palpated by a second rat.

SUMMARY

Eight adult male rats were tested before and after the injection of testosterone propionate for the appearance of feminine mating reactions in response to the copulatory attempts of other males. Prior to hormone administration and after the injection of 6 mg. experimental males usually fought vigorously or tried to escape when other males mounted them in copulatory fashion.

After administration of 13 to 23 mg. of testosterone propionate 5 of the 8 males displayed various elements characteristic of the mating pattern of the estrous female rat. Hopping, lordosis, and vibration of the ears were executed by various injected rats when they were mounted and palpated by other males. The feminine reactions were difficult to elicit and sluggishly performed, but they occurred with sufficient frequency to assure their presence in the behavior repertoire of the experimental animals.

The mating behavior of testosterone treated males is regarded as bisexual rather than homosexual because they displayed very strong masculine copulatory tendencies concomitantly with the appearance of the feminine reactions. It is concluded that the male rat possesses nervous mechanisms capable of mediating the mating pattern of either sex, that testosterone propionate increases the excitability of both patterns, and that within definite limits the stimulus situation is the selective factor determining which type of response will be manifested.

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FRACTIONATION OF NEUTRAL URINARY STEROIDS[1]

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AN EXTENSIVE LITERATURE dealing with the measurement of urinary 17-ketosteroids has accumulated in the past few years. The determinations are ordinarily based upon the color reaction of ketones with m-dinitrobenzene originally described by Zimmerman (1, 2). It has been shown that the 17-ketosteroids give a characteristic color with m-dinitrobenzene (2–5). But according to Holtorff and Koch (6), acetone and other ketones also give the same color. Steroids with carbonyl groups in positions other than the 17-position give a low color titer.

It has indeed been generally recognized that urines contain materials either non-ketonic or non-steroidal that add to the color titer making it "too high" and in many cases lacking any semblance of agreement with biological activity. Several methods have been employed to bring this titer to rational levels. On the assumption that chromogenic pigments are largely responsible for the overestimate various adsorption methods have been resorted to for removing pigment from the neutral fraction containing the ketosteroids (7, 8, 9). We shall present data showing that charcoal adsorbents remove hormone as well as pigment.

Non-specific color may arise from non-ketonic materials in the urine extracts. Accordingly the ketones have been isolated by the use of Girard's reagent T (10). According to these data this has resulted in reducing the apparent 17-ketosteroid titer by as much as 260% in one case but in others by 0 to 30%. The data of Talbot et al. suggest that this decrease in titer ordinarily occurs in urines of low ketosteroid content, since in half of their urine samples which showed no decrease the range of values was 5.0 to 30.0 mg. androsterone equivalent per 24 hours, whereas in the half showing decreases the values ranged from 0.5 to 20.0 mg. The utility of separation of ketones in individual urines is clearly indicated by their data. We shall present data which also indicate decrease in titer when this separation is performed. The titers of urinary 17-ketosteroid reported by various investigators (cf. 10, 11, 12 and others) usually exceed (by 2 to 5 times on the average) the amounts of crystalline material reported isolated (13, 14). This is probably due in part, at least, to losses of material sustained in fractionation and crystallization. Part of the colorimetric titer is due to other ketosteroids which have been recently isolated. Pearlman (15) has isolated aetioallocholanol-3(β)-17-one from normal and pathological urines, but in quantities insufficient to account for any large change in titer. Furthermore this material is in the digitonin-precipitable fraction of urinary ketosteroids and according to Talbot et al. (10) and Baumann and Metzger (12) this fraction contributes from 5 to 15% of the usual titer in normal urines. It should be noted that the bulk of the 17-ketosteroids which have been isolated are alcoholic compounds. Recent evidence indicates that there are non-alcoholic 17-ketosteroids in non-pathological urines. Of such compounds isolated in crystalline form androstenone-17 has been recently identified (14). Chloroandrostenone has been reported (16) in male urine.

In this paper we will present evidence that the non-alcoholic ketonic fraction of normal and pathological urines contains substances which give the Zimmerman color

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reaction and that these substances so contribute to the colorimetric titer as to account for a large part (if not all) of the 'high' values ordinarily encountered.

Colorimetric method (modified Zimmerman reaction). An aliquot of the material to be measured is taken up in 0.2 cc. redistilled 95% ethyl alcohol. To this is added 0.2 cc. of a 2% m-dinitrobenzene solution in redistilled 95% alcohol. The m-dinitrobenzene used is recrystallized from ethanol. Finally, 0.2 cc. of 5 N aqueous KOH is added and the reaction mixture, after thorough mixing, is placed in the dark at room temperature for 45 minutes. At exactly 3 minutes before measurement the sample is diluted with 60% ethanol. Readings are made with a photoelectric colorimeter of our own design, using a Wratten filter having a transmission band at 510 to 520 millimicra. Single measurements are accurate to ±10% and the minimum accurately measurable quantity is 5γ 17-ketosteroid equivalent. A correction for the pigment in highly colored samples is made with aliquots in alcohol to which the usual amount of KOH is added, but the m-dinitrobenzene is omitted. With this technic dehydroisoandrosterone and androsterone give almost equal color titers.

Hydrolysis and extraction of urine samples. The routine method of hydrolysis employed consists of the addition of 15 cc. of concentrated HCl per 100 cc. of urine employed and of boiling for 7 to 10 minutes followed by rapid cooling. The cooled sample is immediately extracted by shaking in a separatory funnel with ether using 4 portions of ether each equal to one-eighth the volume of the urine plus the acid. The combined ether extracts are washed twice with small portions (15 to 20 cc.) of a saturated solution of sodium bicarbonate, once with distilled water, 4 times with 2 N NaOH (to remove the phenolic compounds) and finally with distilled water to remove the alkali.

In testing our fractionation technic, samples prepared by other methods of hydrolysis and extraction were employed. These are referred to in the text.

Fractionation of urine samples. We have developed practicable routine micromethods for the fractionation of the neutral steroids. After some experimentation the following were devised, and, as will be demonstrated in the text, they allow quantitative recoveries of the ketonic steroids.

(*a*) *Micro-Girard separation.*[2] The steroid mixture is thoroughly dried in a pyrex test tube over $CaCl_2$ in a vacuum desiccator; 0.5 cc. glacial acetic acid and approximately 100 mg. Girard's reagent T (trimethyl acethydrazide ammonium chloride) are added. The tube is loosely stoppered with cork wrapped in aluminum foil and placed in an oil bath at 90 to 100° C. for 20 minutes. When the tube is cool, 15 cc. of ice water are added and the reaction mixture is immediately transferred to a small separatory funnel. Sufficient 10% NaOH is added to neutralize nine-tenths of the acetic acid. Three extractions with 20 cc. portions of ether are made. The ether extracts are combined and washed once with 10 cc. ice water. This aqueous wash is combined with the rest of the aqueous ketonic fraction. The ether is washed with 10 cc. 2.5% Na_2CO_3 and with 3 10 cc. portions of H_2O. The ether contains the non-ketonic fraction. The aqueous ketonic fraction is acidified with 3 cc. concentrated HCl, allowed to stand at room temperature for 2 hours, and then extracted 3 times with 20 cc. portions of ether. The ether is washed with 10 cc. 2.5% Na_2CO_3 and 3 times with 10 cc. portions of H_2O. The ether contains the ketonic fraction. This micro-separation should be carried to completion as rapidly as possible since the ketone condensation product will spontaneously hydrolyze if allowed to stand at room temperature.

(*b*) *Separation of alcohols from non-alcoholic compounds.* The steroid mixture is thoroughly dried in a pyrex test tube over $CaCl_2$ in a vacuum desiccator. Approxi-

[2] This is based on a suggestion of Dr. Wolfe cited by Talbot et al. (10).

mately 0.5 gm. succinic anhydride[3] is added, followed by 2 cc. of dry pyridine (dried over BaO, decanted, distilled, and kept over BaO until used). The tube is loosely stoppered with a cork wrapped in aluminum foil and placed in an oil-bath at 90 to 100° C. for 2 hours. The reaction mixture is stirred occasionally. Approximately 10 cc. water are added to the reaction mixture, stirred and permitted to remain in the oil-

TABLE I. ADSORPTION OF 17-KETOSTEROIDS[1]

Exp. No.	Solvent	Androgen	Adsorbent	Amount recovered after adsorption	Recovered after adsorption
		mg.	mg.	mg.	%
1	'Wet'[2] ether	0.200 dehydroisoandrosterone	86 Norite	0.022	11.0
			86 Norite	0.044	22.0
			86 charcoal	0.058	29.0
			86 charcoal	0.074	37.0
2	Dry ether	0.200 dehydroisoandrosterone	86 Norite	0.001	0.5
			86 Norite	0.003	1.5
			86 charcoal	0.000	0.0
			86 charcoal	0.001	0.5
3	CCl_4 dry redistilled	Dehydroisoandrosterone 1.000	40 charcoal	0.082	8.2
		1.000	65 charcoal	0.031	3.1
		0.200	11 charcoal	0.030	15.0
		1.000	81 charcoal	0.021	2.1
		1.000	81 charcoal	0.016	1.6
		0.500	81 charcoal	0.104	20.8
		0.200	81 charcoal	0.039	19.5
		0.200	81 charcoal	0.031	15.5
		0.200	81 charcoal	0.004	2.0
		0.100	81 charcoal	0.004	4.0
		0.050	81 charcoal	0.003	6.0
		0.020	81 charcoal	0.004	20.0
		1.000	40 Norite	0.036	3.6
		0.200	40 Norite	0.036	18.0
		1.000	65 Norite	0.024	2.4
		0.200	65 Norite	0.024	12.0
		1.000	81 Norite	0.002	0.2
		0.200	81 Norite	0.034	17.0
4	'Wet'[2] ether	0.400 dehydroisoandrosterone	86 Norite	0.059	14.8
		0.400 androsterone		0.058	14.5
		0.200 dehydroisoandrosterone plus 0.200 androsterone		0.084	21.0
	'Wet' ether plus neutral fraction of ♂ urine	0.400 dehydroisoandrosterone		0.030	7.3
		0.400 androsterone		0.000	0.0
		0.200 dehydroisoandrosterone plus 0.200 androsterone		0.083	20.8

[1] All materials were dissolved in 30 cc. of the solvent.
[2] U. S. P. Merck.

bath for another half-hour. When the mixture is cool, an extraction is made with 20 cc. then twice with 10 cc. portions of ether. The aqueous residue is discarded. The combined ether layers are washed 4 times with 5 cc. portions of 2 N HCl, then twice with 5 cc. portions of H_2O. The aqueous washes are discarded. The ether solution is extracted with 20 cc. and then 3 times with 5 cc. portions of 0.1 N K_2CO_3 containing 5% NaCl (the NaCl prevents emulsions; more NaCl is added if an emulsion does form). The ether is washed 4 times with 5 cc. portions of H_2O. The ether contains the

[3] Obtained from Eastman Kodak.

non-alcoholic steroids. The K_2CO_3 extract is acidified (to Congo red) with 2 N HCl, and extracted once with 20 cc., then twice with 10 cc. ether. The ether is washed 4 times with 5 cc. portions of H_2O. The ether contains the half-esters of the alcoholic steroids. If desired the free steroids can be obtained by hydrolyzing in alcoholic KOH and then extracting with ether. This step is not really necessary for the determination of alcoholic 17-ketosteroids since the Zimmerman reaction can be directly applied to the succinic acid esters. Higher values are thereby obtained since loss due to an un-

TABLE 2. MODEL EXPERIMENTS ON KETONE SEPARATIONS

Experiment No.	Sample	Dehydroisoandrosterone added	Titer of ketonic fraction	Titer of non-ketonic fraction	Added material recovered
		mg.			%
5	Alcohol solution	0.20	0.20	(0.02)[1]	100.0
4		0.30	0.29	0.00	96.7
3		0.40	0.36	(0.12)	90.0
2		1.00	0.97	0.01	98.0
1		1.00	1.00	0.01	101.0
6		0.00	0.00	0.00	—
7		0.00	0.00	(0.01)	—
8	M.W. ♀ extract of 196 cc. urine	0.00	1.78	(0.15)	—
8		0.00	1.64	(0.14)	—
8		1.00	2.67	(0.17)	96.0
8		1.00	2.83	(0.13)	112.0
9	A.G. ♂ extract of 210 cc. urine	0.00	0.93	(0.15)	—
9		0.00	0.94	(0.16)	—
9		1.00	1.94	(0.16)	100.0
9		1.00	1.88	(0.15)	94.0

[1] Figures in parentheses indicate atypical colors.

necessary manipulation is avoided. The absorption curve for dehydroisoandrosterone is almost identical with that of its succinic acid ester in the modified reaction.

The use of adsorbents. We measured the adsorption of androsterone and dehydroisoandrosterone from solution by Norit A and Merck's activated charcoal. The weighed absorbent was added to 30 cc. of solvent shaken for 3 minutes, filtered at once and washed with 30 cc. of the same solvent in 10 cc. portions. The amount of adsorbent most frequently used (86 mg.) was found to be just sufficient to decolorize an ether solution of one liter equivalent of the neutral fraction from a large sample of pooled male urine. The data summarized in table 1 demonstrate clearly that small amounts of charcoal remove most of these androgens from either CCl_4 or ether. The addition of androgen to a urine extract increased the amount of androgen adsorbed. When we employed ether, which had been dried over sodium and redistilled (*experiment 2*) adsorption was practically complete. The table indicates that there is great variability in the amount of steroid adsorbed. Just what materials adsorbents remove from urine extracts will be indicated below where fractionated urine extracts are considered.

The ketone fractionation. The use of the Girard reagent for obtaining a closer colorimetric estimate of neutral urinary 17-ketosteroids has been recommended (10). In 12 samples these authors recovered 38 to 108% of the 17-ketosteroid titer of unfractionated neutral extracts in the ketonic fraction, whereas in model experiments there was an efficiency of recovery of androgenic ketosteroids of about 93% (17). Our method of fractionation is simpler but no less efficient, as indicated in table 2. *Experi-*

ments 1 to 7 indicate that recovery of pure dehydroisoandrosterone in the amounts employed are 90 to 100% efficient and that in the absence of any hormone the manipulation of the reagents themselves does not contribute to the titer of the ketonic fraction. *Experiments* 8 and 9 on the recovery of dehydroisoandrosterone which had been added to the neutral fraction of a woman's and a man's urine respectively indicate similar efficiency. The 'high' value of 112% in one sample of *experiment* 8 is due to the fact that the amount recovered is determined by subtracting the mean value for the 2 samples to which no dehydroisoandrosterone was added from the sample containing added androgen; the error of the colorimetric determination is obviously enhanced by such subtraction.

We have employed this method of separation on specimens from 10 individuals to demonstrate the extent of chromogen loss as a result of the separation. The data

TABLE 3. THE ZIMMERMAN COLOR TITER OF THE TOTAL NEUTRAL AND THE KETONIC FRACTIONS OF VARIOUS INDIVIDUAL URINE EXTRACTS

Experiment No.	Sample	Total neutral	Ketonic neutral	Ketonic	Method of hydrolysis
		mg.	*mg.*	%	
1	W.H. ♂	12.47	11.59	93.0	Routine
2	S.L. ♂	4.99	4.38	87.8	
3	C.M. ♂	20.16	12.80	63.6	
4	M.H. ♂	3.48	2.14	61.5	
5	C.L. ♀	2.63	2.00	76.0	
6	M.H. ♀	11.60	8.35	72.0	
7	A.B. ♀	2.26	1.56	69.0	
8	L.D. ♀	1.28	0.56	43.7	
9	T.F. ♂	2.60	1.73	66.6	Looney
9	T.F. decolorized	1.52	1.00	65.8	
10	E.B. ♂	2.39	1.44	60.3	
10	E.B. decolorized	1.46	0.69	47.3	

are present in table 3. The first 8 specimens were hydrolyzed by our routine method. The next 2 were kindly supplied by Dr. Joseph Looney of the Worcester State Hospital, and were treated by him as follows: (*a*) brought to pH 1.0 with sulfuric acid, (*b*) autoclaved at 15 lb. pressure for 15 minutes, (*c*) cooled and extracted by redistilled benzene for 5 hours in a Koch continuous extractor, (*d*) the benzene distilled off in vacuo and the residue taken up in ethyl ether, (*e*) the ethereal solution extracted with 20% Na_2CO_3 and an ether backwash of the Na_2CO_3 added to the ethereal solution, (*f*) the ethereal solution washed with 1 N NaOH followed by washing with 20% sodium bisulphite and then with distilled water, (*g*) the ether extract (at this point about 60 cc. in volume) was divided into 4 equal portions, one of which was decolorized with Norit A (not more than 125 mg.) and is denoted as decolorized in the table, a second was used for colorimetric determination for comparison with the decolorized sample, after both were subjected to the Girard separation.

The data of table 3 confirm Talbot et al. (10) in their finding that certain urine extracts suffer a loss in colorimetric titer after removal of the non-ketonic materials. In this series losses up to 56% of the titer of the total neutral extract were encountered. Secondly the data on the urines hydrolyzed by the Looney method demonstrate that the decolorization procedure removes non-ketonic and ketonic chromogens. There appears to be no regular relation between the titer of the total neutral material and its ketonic fraction.

Separation of alcoholic ketosteroids. In order to establish quantitative recoveries the experiments of table 4 were instituted. In series *A* and *B* the succinic acid ester was hydrolyzed and the free hormone recovered. In series *C* and *D* the colorimetric measurements were made upon dehydroisoandrosterone succinate, m.p. 267°–268° C. and the concentrations determined from a standard curve obtained with the succinate. *Experiments A* and *B* demonstrate that 75% to 93% (average 84.8%) of the dehydroisoandrosterone is recovered in the alcoholic fractions, and 3 to 16% (average 7.1%)

TABLE 4. SUMMARY OF DATA ON SUCCINIC ANHYDRIDE SEPARATIONS

Sample No.	Amt. of dehydroisoandrosterone	Recovered in alcoholic fraction	Recovered in non-alcoholic fraction	Amt. in alc.	Amt. in non-alc.	Alcoholic as % of total recovered	Amt. lost
	mg.	mg.	mg.	%	%		%
1A	3.00	2.28	0.28	76.0	9.3	89.0	14.7
2A	3.00	2.68	0.10	87.0	3.3	96.4	9.7
3A	1.00	0.74	0.09	74.0	9.0	89.2	17.0
4A	1.00	0.82	0.07	82.0	7.0	92.3	11.0
5A	0.50	0.42	0.08	84.0	16.0	84.0	0.0
6A	0.50	0.45	0.02	90.0	4.0	95.8	6.0
7A	0.00	(0.01)[1]	0.00				
8A	0.00	(0.01)	0.00				
1B	3.00	2.63	0.11	87.7	3.7	96.0	8.6
2B	3.00	2.78	0.09	92.6	3.0	97.0	4.4
3B	0.60	0.48	0.03	80.0	5.0	94.2	15.0
4B	0.60	0.54	0.03	90.0	5.0	94.8	5.0
5B	0.20	0.16	0.02	80.0	10.0	88.8	10.0
6B	0.20	0.15	0.02	75.0	10.0	83.3	15.0
7B	0.00	0.00	0.00				
8B	0.00	0.00	(0.01)				
1C	0.20	0.165	0.021	82.5	10.5	88.7	7.0
2C	0.20	0.179	0.021	89.5	10.5	89.5	0.0
3C	0.10	0.091		91.0			
4C	0.10	0.093		93.0			
5C	0.05	0.045		90.0			
6C	0.05	0.046		92.0			
1D	1.00	1.06		106.0			
2D	1.00	1.02		102.0			
3D	0.00	(0.02)	(0.01)				
4D	0.00	(0.02)	(0.01)				
5D	1D non-alcoholic	(0.06)	(0.02)				
6D	2D non-alcoholic	(0.08)	(0.03)				

[1] Figures in parentheses indicate atypical colors.

remain in the non-alcoholic fractions. When we calculate the alcoholic fraction titers as a percentage of the total titers, the data show 83 to 97% (average 91.5%) of the material is in the alcoholic fractions. The percentage of material lost in the manipulations varies from 0 to 17% (average 9.7%). The data of *experiments C* and *D* indicate that if the hydrolysis of the half-ester is not carried out the percentages of material recovered in the alcoholic fractions is higher varying from 83 to 106% (average 93.3%). The indications from the non-alcoholic fraction titers of *experiments A* and *B* are that 7% of the dehydroisoandrosterone fails to esterify (or be extracted by the K_2CO_3); this is substantiated by the 93% recovery of alcoholic material in *experiments C* and *D*. The blank experiments *7B*, *8B*, *3D*, *4D*, *5D*, *6D* indicate no or negligible contributions to the titer by the reagents employed.

TABLE 5. RECOVERY EXPERIMENTS WITH DEHYDROISOANDROSTERONE AND ANDROSTERONE

Exp. No.	Sample	Hormone added	When added	Titer of alcoholic fraction	Titer of non-alcoholic fraction	Total	Recovered in alcoholic fraction	Recovered in non-alcoholic fraction	Total recovered
				mg.	mg.		%	%	%
1	500 cc. tap water	2mg. dehydroisoandrosterone	Before hydrolysis	0.13[1]	0.72[2]	0.85	6.5	36.0	42.5
		2 mg. dehydroisoandrosterone		0.12[1]	0.67[2]	0.79	6.0	33.5	39.5
		2 mg. androsterone		1.13[1]	0.30[1]	1.43	56.5	15.0	71.5
		2 mg. androsterone		0.96[1]	0.35[1]	1.31	48.0	17.5	65.5
2	500 cc. from pooled male urine			2.75	2.41	5.16			
				2.70	2.35	5.05			
		2 mg. dehydroisoandrosterone	After (1) hydrolysis	4.24	2.13	6.37	75.5	(−13.0)	(62.5)
		2 mg. androsterone	After (1) hydrolysis	4.65	2.39	7.04	96.0	0.0	96.0
		2 mg. dehydroisoandrosterone	Before hydrolysis	3.28	3.25	6.53	27.5	43.0	70.5
		2 mg. androsterone	Before hydrolysis	3.69	3.50	7.19	48.0	55.5	103.5
3	500 cc. from pooled male urine			2.88	1.66	4.54			
				2.69	1.57	4.36			
		2 mg. dehydroisoandrosterone	After (2) hydrolysis	4.99	1.71	6.70	110.0	2.0	112.0
		2 mg. androsterone	After (2) hydrolysis	4.39	1.80	5.75	80.0	6.5	86.5
		2 mg. dehydroisoandrosterone	Before hydrolysis	3.14	1.89	6.25	17.5	11.0	28.5
		2 mg. androsterone	Before hydrolysis	3.54	2.42	5.96	37.5	37.5	75.0
4	500 cc. from pooled male urine			2.67	1.59	4.26			
				2.94	1.80	4.74			
		2 mg. dehydroisoandrosterone	After (2) hydrolysis	4.65	1.47	6.12	92.0	(−11.0)	(81.0)
		2 mg. dehydroisoandrosterone	After (2) hydrolysis	4.79	1.69	6.48	99.0	0.0	99.0
5	500 cc. tap water	3.45 mg. dehydroisoandrosterone succinate	Before hydrolysis	0.18	1.11	1.29	7.0	43.0	50.0
		3.45 mg. dehydroisoandrosterone succinate	Before hydrolysis	0.38	1.42	1.80	12.8	55.0	70.0
6	500 cc. from pooled male urine			2.85	1.70	4.55			
				2.66	1.54	4.20			
		3.45 mg. dehydroisoandrosterone succinate	Before hydrolysis	3.01[1]	2.69[2,3]	5.70	9.7	41.5	51.2
		3.45 mg. dehydroisoandrosterone succinate	Before hydrolysis	3.39[1]	3.51[2]	6.90	24.4	73.3	97.7

[1] Negative Rosenheim reaction.
[2] Positive Rosenheim reaction.
(1) Added to the acid urines.
(2) Added to the ethereal extracts of the acid urines.
[3] May have been some loss of material in extraction.

The efficacy of the extraction procedures and of the half-ester separation was tested by adding known amounts of dehydroisoandrosterone and androsterone to tap water and to aliquots of samples of pooled male urine. The data of these recovery experiments are presented in table 5. Our routine method of hydrolysis was employed. In the case of the urine samples, the mean titers of the extracts of the aliquots con-

TABLE 6. VARIATIONS IN METHOD OF HYDROLYSIS AND ALCOHOLIC: NON-ALCOHOLIC 17-KETOSTEROID TITERS

Exp. no.	Sample	Alcoholic	Non-alcoholic	Total	Non-alcoholic	Method of hydrolysis
		mg.	*mg.*	*mg.*	%	
1	500 cc. from pooled male urine	2.80	2.21	5.01	44.1	Routine
1	500 cc. from pooled male urine	2.89	2.11	5.00	42.5	Routine
1	500 cc. from pooled male urine	3.45	1.30	4.75	27.4	Callow
1	500 cc. from pooled male urine	3.72	1.89	5.61	33.7	Callow
2	1 liter from pooled urine[1]	6.15	10.52	16.67	63.2	Routine
2	1 liter from pooled urine[2]	7.61	7.13	14.74	48.3	Lawrence and Werthessen
2	1 liter from pooled urine[2]	3.85	3.10	6.95	44.6	Simultaneous extraction and hydrolysis
2	1 liter from pooled urine[2]	4.34	2.45	6.79	36.1	Simultaneous extraction and hydrolysis
3	♂ *H.S.* decolorized	4.05	1.38	5.43	24.8	Looney
4	♂ *H.S.* decolorized	7.92	1.24	9.16	13.5	Looney
5	♂ *S.A.* decolorized	5.21	1.10	6.31	17.4	Looney
6	♂ *H.T.* decolorized	2.33	0.52	2.85	19.2	Looney
7	♂ *E.B.* decolorized	2.60	0.54	3.14	17.2	Looney
8	♂ *L.C.* decolorized	2.29	1.18	3.47	34.0	Looney
9	♂ *L.C.* before decolorizing	1.92	3.20	5.12	62.5	Looney
9	♂ *L.C.* after decolorizing	1.84	1.10	2.94	37.4	Looney
10	♂ *H.T.* before decolorizing	4.08	3.96	8.04	48.3	Looney
10	♂ *H.T.* after decolorizing	4.08	1.62	5.70	28.4	Looney
11	♂ *T.F.* before decolorizing	4.79	4.12	8.91	46.8	Looney
11	♂ *T.F.* after decolorizing	4.69	1.85	6.54	28.3	Looney
12	♂ *H.T.* before decolorizing	8.13	7.91	16.04	49.3	Looney
12	♂ *H.T.* after decolorizing	3.69	1.68	5.37	31.3	Looney
13	♂ *P.S.* before decolorizing	7.08	4.55	11.63	39.1	Looney
13	♂ *P.S.* after decolorizing	5.00	1.71	6.71	25.5	Looney
14	0.50 mg. dehydroisoandrosterone in tap water	0.50	(0.03)	0.50		No hydrolysis
14	0.50 mg. dehydroisoandrosterone in tap water	0.42	0.06[2]	0.48	12.5	1% HCl boiled for 15 min.
14	0.50 mg. dehydroisoandrosterone in tap water	0.24	0.17[3]	0.41	41.5	5% HCl boiled for 15 min.
14	0.50 mg. dehydroisoandrosterone in tap water	0.11	0.22[3]	0.33	66.6	10% HCl boiled for 15 min.

Figures in parentheses indicate atypical colors.
[1] Hydrolyzed 2 days after other samples.
[2] Negative Rosenheim reaction.
[3] Positive Rosenheim reaction.

taining no added hormone were subtracted from the titers of the extracts containing added hormone in order to obtain an estimate of the amounts recovered. Two important deductions from table 5 are: (*a*) the hydrolysis we employ results in the loss of considerable amounts of dehydroisoandrosterone and lesser but appreciable amounts of androsterone, (*b*) the hydrolysis we employ leads to the recovery of most of the added dehydroisoandrosterone in the non-alcoholic fraction and a lesser but considerable conversion of androsterone to non-alcoholic material. In the case of the free hormones (*experiments* 1 to 3) from 28.5% to 70.5% of added dehydroisoandrosterone subjected to hydrolysis is recovered as 17-ketosteroid; the corresponding percentages of androsterone are 65.5% and 103.5%. But the data of these

experiments indicate that in the case of dehydroisoandrosterone as much as 80% of the 17-ketosteroid may be converted to non-alcoholic material, and for androsterone almost 50%. The non-alcoholic fractions from the dehydroisoandrosterone hydrolysates of *experiments* 2 and 6 of table 5 and *experiment* 14 of table 6 gave positive Rosenheim reactions whereas negative Rosenheim reactions were obtained in the non-alcoholic fractions from the hydrolysates of androsterone (table 5). The Rosenheim reaction is presumably specific for doubly unsaturated compounds of the type of $\Delta 3:5$ androstadienone-17.

The hormones are predominantly excreted as esters (not yet identified chemically). Dehydroisoandrosterone succinate was added to tap water and urine in the hope of imitating the effects of hydrolysis on the urinary ester. In these experiments (Nos. 4 and 5) we obtained a larger recovery of total 17-ketosteroid titer (i.e., less destruction by acid hydrolysis) but a nonetheless large conversion to non-alcoholic material (75 to 85% of recovered material in the non-alcoholic fractions). The efficiency of the separations was in each instance checked by hormone added to the neutral fractions after hydrolysis; the recoveries were about the same as those for pure materials (cf. table 4).

In table 6 we present further evidence that the titer of the non-alcoholic fractions is governed by the method of hydrolysis employed. In *experiment* 1 a comparison is made of the 17-ketosteroid titer of the non-alcoholic fractions when our routine hydrolysis is followed and that of Callow et al. (18) using identical urine samples. By our method 43.3% of the titer is in the non-alcoholic fraction, by the Callow method 30.6% is in the non-alcoholic fraction. In *experiment* 2, one liter aliquots of a pooled urine from various sources was treated as follows: (*a*) pH adjusted to 1.0 with HCl, brought to a boil and then placed in a continuous extractor for 2 hours using toluene as the solvent [method of Lawrence and Werthessen (19)], (*b*) pH adjusted to 1.0 with HCl, placed while cold in the continuous extractor with toluene, (*c*) routine hydrolysis. The non-alcoholic titers were respectively 48.3%, 40.3% and 63.2% of the total. It should be noted, however, that larger total recoveries were obtained by the Lawrence and Werthessen method and by our routine hydrolysis and extraction.

A fourth method, that of Dr. Looney, gave in a series of decolorized samples (Nos. 3 to 8) 13.5% to 34.0% of the titer in the non-alcoholic fractions. These low titers led us to examine a set of 5 specimens supplied by Dr. Looney and divided into decolorized and non-decolorized aliquots. The data on these samples (Nos. 9 to 13) show 39.1% to 62.5% (average 49.2%) of the titer in the non-alcoholic fractions before decolorizing and 25.5% to 37.4% (average 30.2%) after decolorizing. Decolorizing charcoal therefore tends to remove non-alcoholic materials rather than alcoholic compounds though the data do show that in certain instances both were removed in large amount (No. 12 especially).

Experiment 14 of table 2 demonstrated that when pure dehydroisoandrosterone is added to tap water the conditions of hydrolysis determine the extent of conversion into non-alcoholic substance. The titer of non-alcoholic material rises from 12.5% of the total to 66.6% as the concentration of HCl is increased from 1% to 10%. It should be noted too that increasing the acid concentration causes increased destruction of 17-ketosteroid material.

Nature of the chromogenic material in urine extracts. Through the courtesy of Dr. N. T. Werthessen of the Endocrine Laboratory of the Boston Dispensary, we obtained absorption spectra of the Zimmerman color reaction of various fractions from a large sample (188 liters) of pooled male urine (20). In figure 1 we present the absorption curves for 25 cc. of urine equivalent for (A) the total neutral fraction, (B) the ketonic neutral fraction, (C) the non-alcoholic ketonic fraction, (D) the alcoholic ketonic fraction and (G) the non-ketonic fraction of this large sample. As shown

by Talbot et al. (10) the non-ketonic fraction exhibits an absorption curve quite unlike that of the ketonic fractions. The other fractions show the typical absorption peak at 510 to 520 millimicra, but when comparison is made with the absorption curve of pure dehydroisoandrosterone, (E) it is obvious that there is a larger absorption in the ultra-violet and violet in the urine extracts. The ratio of the absorptions at the peaks (region of 520 $\mu\mu$.) to the absorption at 420 $\mu\mu$. is as follows: dehydroisoandrosterone, 2.0; total neutral, 1.4; ketonic neutral, 1.4; non-alcoholic ketonic, 1.3;

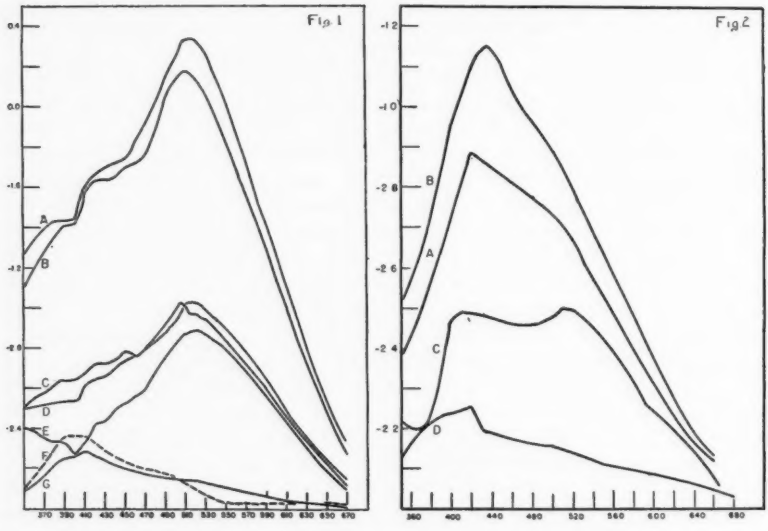

Fig. 1. Absorption curves of the colored compounds produced in the Zimmerman reaction of (A) the total neutral, (B) the ketonic neutral, (C) the non-alcoholic ketonic neutral, (D) the alcoholic ketonic neutral, (G) the non-ketonic neutral fractions of normal male urine. Each curve represents an extract aliquot equivalent to 25 cc. of urine. Curve E is the absorption curve of dehydroisoandrosterone and curve F is derived by subtracting E from C. Abscissa = wavelength in millimicra, ordinate = optical density (log 1/transmission).

Fig. 2. Absorption curve of the colored compounds produced in the Zimmerman reaction of (A) a chromatographed fraction of the non-alcoholic ketonic material of women's urine, (B) the same fraction of cancerous women's urine, (C) the non-alcoholic ketonic fraction from unhydrolyzed fresh male urine and (D) cholestenone (100 γ). Abscissa = wavelength in millimicra, ordinate = optical density (log 1/transmission).

alcoholic ketonic, 2.0. All of the material giving an atypical color reaction appears, therefore, to be confined to the non-alcoholic fraction. The curve F represents the calculated absorption curve of the atypical material in the non-alcoholic fraction. In figure 2 we present the Zimmerman reaction absorption spectra of (A) a chromatographed fraction of the non-alcoholic ketones of pooled female urine and (B) a similar fraction from the pooled urines of a group of cancerous women, (C) the total non-alcoholic neutral fraction of an extract of fresh unhydrolyzed normal male urine and (D) of cholestenone for comparison. It can be seen that A and B resemble D and that C might very well represent a mixture of the 17-ketosteroid and 3-ketosteroid colors. The absorption spectrum of the material of the non-alcoholic fraction of unhydrolyzed urine differs markedly from that of the same fraction from hydrolyzed urine (fig. 1 C).

DISCUSSION

In this paper we have presented what we consider to be practicable routine methods for the separation of the neutral steroids into (*a*) ketonic and non-ketonic and (*b*) alcoholic ketonic and non-alcoholic ketonic fractions. We have shown that (*a*) is useful inasmuch as it removes definite quantities of chromogenic non-ketonic materials. The usefulness of (*b*) resides in the fact that it segregates the hydroxy ketones (of which androsterone and its isomers and dehydroisoandrosterone are representative) from the non-hydroxylated compounds. We may properly speak of the hydroxy-ketonic fraction as containing a true titer of 17-ketosteroids since its absorption curve appears to be practically that of dehydroisoandrosterone (and androsterone) whereas there is good evidence that a portion, at least of the non-alcoholic ketonic fraction is not 17-ketosteroid. As to the material contributing to the true 17-ketosteroid titer of the non-alcoholic fraction we believe it to include androstenone-17, $\Delta 3:5$ androstadien one-17, and possibly chloroketones derived from androsterone, its isomers, and dehydroisoandrosterone. Thus far Δ 3:5 androstadienone-17 has been isolated only from urines from pathological cases (20, 22) but it probably occurs in small amounts normally as an artifact of the hydrolysis of dehydroisoandrosterone (23).

Although we have presented clear evidence that acid hydrolysis causes the conversion of androsterone and dehydroisoandrosterone to non-alcoholic substances it does not follow that all of the ketosteroid in the non-alcoholic ketonic fraction necessarily arises as hydrolysis artifact. We have presented evidence that non-alcoholic material occurs in fresh unhydrolyzed men's urine in an amount equal to about 10% of the amount of non-alcoholic material obtained after acid hydrolysis of the residual urine (23). A repetition of this with a sample of women's urine gave the same proportions (0.38 mg. 17-ketosteroid equivalent from the unhydrolyzed urine, 4.01 mg. from the residual urine after acid hydrolysis). Further, while it is hard to visualize the possible conjugated form of a non-alcoholic ketosteroid in urine, such compounds may exist and account in part for the titer of the non-alcoholic urine fractions. Nonetheless a definite moiety of this titer must be due to artifacts arising from the action of HCl on androsterone and dehydroisoandrosterone.

SUMMARY

A simple micromethod, using Girard's reagent T, is described whereby the neutral urinary steroids are separated into ketonic and non-ketonic fractions.

Non-ketonic chromogens may contribute considerably to the 17-ketosteroid colorimetric titer of individual urinary specimens. Removal of such chromogenic material with the aid of charcoal adsorbents will result in the simultaneous removal of variable amounts of 17-ketosteroids.

The neutral 17-ketosteroids may be quantitatively resolved into alcohols and non-alcohols by a simple microtechnic involving half-esterification with succinic anhydride.

Acid hydrolysis causes a large conversion of dehydroisoandrosterone to non-alcoholic material, but androsterone is less labile. It seems likely, therefore, that other urinary hydroxyketones are similarly affected.

The non-alcoholic ketonic fractions of pooled urines contain appreciable amounts of material which give an atypical Zimmerman color reaction whereas such material is absent from the alcoholic ketonic fractions.

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ADDENDUM

While this paper was in press a paper by Talbot et al. (J. Biol. Chem. 139: 521. 1941) appeared describing a chromatographic method for separating the non-alcoholic from the alcoholic 17-ketosteroids. The Zimmerman color titer of their non-alcoholic fractions never exceeded 9% of the titer of the total neutral ketonic fraction. With aliquots of 5 urine samples we have compared the colorimetric titer obtained by their method of simultaneous hydrolysis and CCl_4 extraction and our routine method of independent hydrolysis and ether extraction. Two obvious differences were observed. (1) Recoveries of total ketonic material by our method were approximately twice those obtained by the method of Talbot et al. (2) The ratios of non-alcoholic to alcoholic material obtained by the succinic anhydride treatment were nearly identical by the two methods of hydrolysis and extraction, ranging from 1:5 to 1:0.8 in individual samples. We attribute our higher total titers to the use of ether as an extractive. Since these data exclude the method of independent hydrolysis and extraction as the possible cause of the higher titer of non-alcoholic material obtained, we are inclined to believe that the chromatographic method involves the differential adsorption of certain non-alcoholic chromogens (perhaps those giving atypical color) that are not lost by the chemical methods we employ. The net result is that the method of Talbot et al. probably gives an overestimate of the alcoholic 17-ketosteroid titer since they determine it by subtracting the titer of their eluted 'non-alcoholic' fraction from the titer of the total ketonic material. We believe therefore that our chemical method of separation is preferable. In addition, the variability of an adsorption method (see Talbot et al., footnote page 524) and the scarcity and expensiveness of the Brockman alumina recommended offer serious drawbacks.

EFFECT OF TESTOSTERONE PROPIONATE ON BONE GROWTH AND SKELETAL MATURATION OF NORMAL AND CASTRATED MALE RATS.[1,2]

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THE ACTION of testosterone propionate consists fundamentally in increasing the growth and development of genital structures in the male, including the accessory sexual characteristics. Aside from this highly specialized effect, it is reasonable to assume that other components of the body may also be influenced. A number of experimental studies in animals and man have been performed with regard to possible measurements of such responses to androgens. Kochakian (1) and Kenyon et al., (2) demonstrated a retention of nitrogen and sodium during testosterone treatment. Hesser and his associates (3) have noted that androgenic treatment produced changes in myotonic dystrophy when associated with eunuchoidism. Rubenstein and Solomon (4, 5) found that 0.05 mg. of testosterone administered daily to growing albino rats led to a "probable" increase in body weight and length, whereas, 1 mg. daily suppressed these same responses in castrate animals to a still greater degree than castration alone. Rapfogel (6) believed that possibly the androgens may influence bone growth, but felt that potent androgenic therapy may be antagonistic to the growth stimulant effect of the anterior pituitary. McCullagh and McGurl (7) reported an increase in the rate of epiphyseal union in some of their patients.

These reports have led to the inference that testosterone definitely influences general skeletal growth and maturation. In the present study, an attempt has been made to measure more exactly the effects of this androgen on osseous development in the male rat.

EXPERIMENTAL METHODS

Sixteen litters representing 86 male rats were used. These were segregated into 8 groups, depending upon the time and amount of treatment, and whether castrate or intact.

Group A, 30 rats, served as controls, and consisted of litter mates of those assigned to *groups B, C* and *D*.

Group B, 10 rats, received the daily equivalent of 0.25 mg. of testosterone propionate in sesame oil. They were injected subcutaneously, every other day, beginning at 25 days of age and continuing for a total of 125 days.

Group C, 11 rats, received the equivalent of 0.25 mg. of testosterone propionate daily for 80 consecutive days. Injections were begun when the rats were 1 day old.

Group D, 10 rats, were treated with 1.0 mg. of the androgen, beginning at 1 day of age and continuing for 100 days.

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[2] Presented before the 24th annual meeting of the Association for the Study of Internal Secretions, New York City, 1940.
Acknowledgment is made of the valuable technical assistance of Miss Ada Baughn.

Group E, 7 rats, were castrated when 1 day old and were controls for *group F*.

Group F, 7 rats, were castrated the first day after birth. Following a 5-day recovery period, daily injections of 0.25 mg. of androgen were begun and continued for 135 successive days.

Groups G and H, representing respectively 5 controls and 6 experimentals, were injected from date of birth with 2 mg. of testosterone propionate daily for 16 days.

A summary of the 8 groups of rats and the manner of treatment is listed in table 1.

TABLE 1. SUMMARY OF THE AMOUNT AND TIME OF TESTOSTERONE INJECTIONS

Group	No. rats	Testosterone	Age when treatment started	Length of treatment	Age at autopsy	Remarks
		mg./day	*days*	*days*	*days*	
A	30	None	—	—	150	Controls; litter mates of of *groups B, C, D*
B	10	0.25	25	125	150	Intact animals
C	11	0.25	1	80	115	Intact; treatment from first day after birth
D	10	1.0	1	100	100	Intact animals
E	7	None	—	—	135	Castrated when 1 day old and controls for *group F*
F	7	0.25	5	135	135	Castrated on first day of life; treatment begun on day 5
G	5	None	—	—	20	Controls for *group H*
H	6	2.0	1	16	20	Intact

The control animals, *groups* A, *E* and G, were injected with sesame oil in equal quantity to that used in the testosterone-treated rats. All animals were weighed and measured weekly throughout the experimental period. Body lengths were recorded as the distance between anus and tip of snout. Roentgenograms were made at intervals of 1 to 10 days on both controls and treated animals to a total of 600 in this series. Careful attention was given to osseous development, using the following criteria.

First appearance of: Carpal and tarsal bones, patella and sesamoid bones around knee, and the os penis, epiphyses for distal and proximal ends of tibia, for distal end of humerus and femur, for proximal end of ulna and radius, for posterior end of calcaneus, for ends of metacarpals and phalanges, and for ischial tuberosities.

Fusion of: Head and shaft of radius, epiphysis of olecranon to shaft of ulna, epiphysis of calcaneus to calcaneus, epiphyses of metacarpals and metatarsals to shafts.

Body weight and length, and length of femora and humeri.

At termination of the experiment, the rats in each group were killed, femora and humeri dissected, x-rayed for comparisons of density and degree of epiphyseal fusion, and measured for variations in length.

RESULTS

Careful analysis of results showed but two significant features, namely, the time of the first appearance and rate of development of the os penis and of the epiphyses for the ischial tuberosities. The appearance and fusion of other skeletal structures were not noticeably influenced, even when as much as 2 mg. testosterone propionate were injected daily, beginning at time of birth. Neither did the treatment significantly alter body weight and length, nor the length of femora or humeri.

The os penis. This could not be positively identified in any of the groups until the animals approached the 20th day of age.

At this age, it may be seen in figure 1, that the os penis was present, as indicated by roentgenograms, in 5% of the untreated unoperated animals (*group* A). At the age of 50 days, 87% had developed the structure, and by 71 days after birth its presence was established in all animals.

In the treated but unoperated animals (*groups B, C, D*), no significant differences were observed among the 3 groups, regardless of the dosage used or age of the rats at the time treatment was begun. The data are presented as a combined curve in figure 1. It will be seen, in those animals injected from day of birth, the os penis was definitely developed in 13% of the animals at the age of 20 days. Within the next 35 days, rapid progress was shown, the os penis being demonstrable in all animals 55 days after birth.

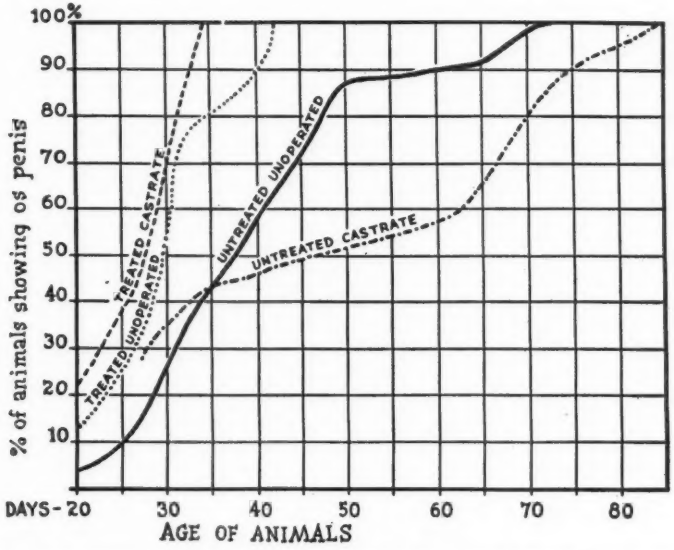

Fig. 1. The influence of testosterone propionate on the growth response of the os penis in male rats. Note acceleration in treated animals.

The untreated castrate animals (*group E*) were 28 days old before the os penis could be identified and then in only 2 rats. The rate of appearance was also delayed, and a lapse of 84 days ensued before 100% of the rats showed the mature os penis.

In the treated castrates (*group F*), 22% of the animals showed the os penis at 20 days of age, and the process was completed in all rats by the 34th day of life. This represents a definite acceleration.

Ischial tuberosities. A summary of the observations is presented in figure 2. The untreated unoperated controls showed development of the epiphysis in 1 animal only at 59 days of age. Progress was rapid during the next 28 days, and all animals presented clearly defined epiphyses at 87 days.

In animals in the treated unoperated groups, again shown as a combined curve in figure 2, it became apparent 48 days after birth that the epiphyses were appearing at a faster rate than in the controls, and 30% presented well defined epiphyses at this time. The process was completed within 2 weeks, or by the age of 62 days.

In the untreated castrates, the animals were 62 days old when 25% of the group showed developing epiphyses. On the 82d day after birth, epiphyses were observed in all animals.

The greatest acceleration appeared in the treated castrate animals. About half of of the animals revealed the epiphyses at 50 days of age, and the process was completed in all animals when 67 days old.

Statistical treatment of the data obtained from the intact animals in *groups A, B, C* and *D*, indicates a significant difference in the time of appearance of both the os penis and epiphyses for the ischial tuberosities in the hormone-treated animals as compared with the controls (table 2). The degree of significance was obtained by employing the formula, $t = \frac{M_1 - M_2}{\sqrt{E_1^2 + E_1^2}}$.

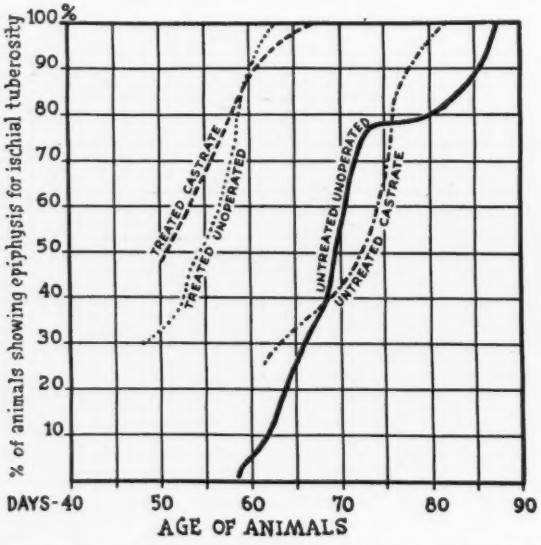

Fig. 2. THE INFLUENCE OF TESTOSTERONE PROPIONATE ON THE RATE OF DEVELOPMENT OF THE EPIPHYSES OF THE ISCHIAL TUBEROSITIES IN MALE RATS. Note acceleration in treated animals.

TABLE 2. TIME OF APPEARANCE OF OS PENIS AND EPIPHYSES FOR ISCHIAL TUBEROSITIES IN INTACT ANIMALS

	Average time of appearance				$(t)^2$
	Control		Treated		
	No. of rats	Days	No. of rats	Days	
Os penis	30	40±2.4[1]	31	31±1.1	3.3
Epi. ischial tuberosity	30	72±1.4	31	54±2.0	7.3

[1] Standard error.
[2] Degree of significance.

DISCUSSION

With the exception of the observed acceleration in development of the os penis and in epiphyses for the ischial tuberosities, definite changes could not be demonstrated. In this respect, the constancy of the gain in weight and length in all animals whether treated or untreated is of interest in view of the published reports of Rubenstein and Solomon already mentioned. Even those animals which received 1 mg. daily for 100 consecutive days and were treated from the first day of birth showed no significant changes when compared with the control group.

The possibility that large doses of testosterone propionate may increase the rate of osseous maturation was excluded by the following observations. The dissected humeri and femora of both the treated and control animals were measured and x-rayed to demonstrate differences in density and to evaluate the epiphyseal status. Although

there were individual variations in length, skeletal maturation and density of bones, these were no greater in amount in the treated than in the control animals.

In regard to epiphyseal closure, it must be remembered that in the rat the epiphyses in certain locations fail to fuse, and this condition was not altered by the administration of testosterone propionate.

The failure of testosterone propionate to accelerate calcification is in agreement with the observations of Wentworth et al., (8) but stands in marked contrast to the

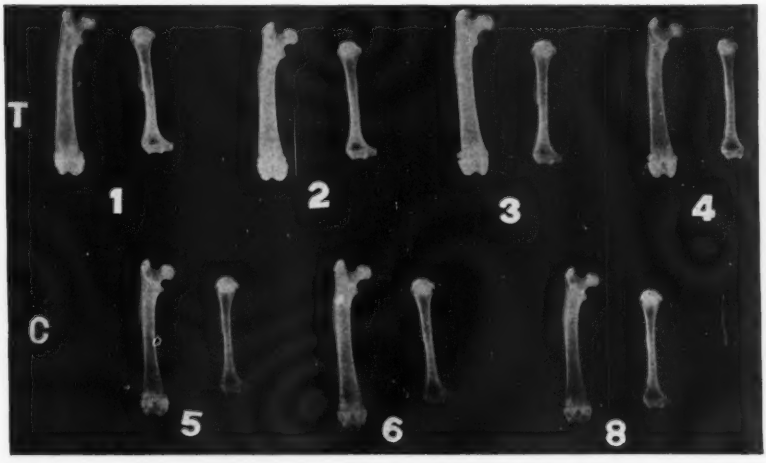

Fig. 3. Roentgenograms of femora and humeri of treated rats (T) and controls (C) show no difference in density or length of bones nor in the degree of epiphyseal fusion.

reports of the same authors for the estrogens which increase deposition of bone in the marrow cavities.

SUMMARY

Prolonged injections of large amounts of testosterone propionate, even when begun one day after birth, did not significantly alter skeletal maturation or body growth in rats. Appearance of the os penis and of the epiphyses for the ischial tuberosities was hastened. However, osseous progress of the untreated animals was such that at 87 days of age no skeletal difference between control and treated rats existed, whether castrated or intact, even with regard to the os penis and the epiphyses for the ischial tuberosities.

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INFLUENCE OF ADRENAL PREPARATIONS ON BASAL METABOLISM AND SPECIFIC DYNAMIC ACTION[1]

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OVER A PERIOD of years evidence has been accumulating for the participation of the adrenal cortex in the maintenance of the normal metabolic rate. Among the first to furnish conclusive proof were Marine and Baumann (1) and Aub, Forman and Bright (2) who observed a fall in metabolism after adrenalectomy.

Swingle et al. (3) demonstrated that cortical extract would return the basal metabolism of adrenalectomized cats to normal. At about the same time this was also shown by Hartman et al. (4) in rats. More recently Thorn and his associates (5) found no change in the metabolism of adrenalectomized dogs kept in good condition by desoxycorticosterone acetate. They studied the fasting blood sugar, the tolerance curve following oral glucose administration, the fasting respiratory quotient (R.Q.) and the R.Q. following glucose administration. No change was found in any of these values. It is interesting to note that in patients with Addison's disease Thorn et al. (6) found a low blood sugar curve following orally administered glucose. Prolonged treatment with desoxycorticosterone acetate corrected the fasting hypoglycemia and brought about some improvement in the dextrose tolerance curve. Patients with Addison's disease frequently showed marked hypoglycemia 2 to 3 hours after intravenous administration of glucose. Desoxycorticosterone acetate therapy ameliorated the symptoms of hypoglycemia but did not affect the blood sugar level.

Opinion seems to be divided concerning the effect of adrenalectomy on specific dynamic action (S.D.A.). On the one hand, Nord and Deuel (7) stated that no change was found in S.D.A. after 10 gm. of glycine was administered either orally or intravenously to adrenalectomized dogs. However, our calculation from their figures showed approximately an 18% decrease. Their adrenalectomized dogs showed significant rises in basal metabolism over the controls which is contrary to the findings of other workers. Moreover, the control dogs were not those used for adrenalectomy. Arvay (8) on the other hand, observed a 50% decrease in S.D.A. of rats showing symptoms of insufficiency. His animals showed the fall of basal metabolism now known to be characteristic of adrenalectomized animals. His experiments were better controlled in that determinations were made on the same animal before and after adrenalectomy. Arvay studied the S.D.A. of 3 gm. of meat powder mixed with 1 gm. of fat administered orally.

Recently, it has been shown that adrenal extracts can be divided into two fractions (9), one which is concerned with sodium metabolism and the other which maintains adrenalectomized animals in good condition but is without effect on sodium metabolism. The essential substance in the first fraction has been called the sodium factor while that in the second has been called cortin. Since it had been well established that experimental adrenal insufficiency caused a fall in basal metabolism and that adrenal extract essentially adrenaline-free returned the basal metabolic rate to

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normal, it was deemed worthwhile to test the various fractions which we had obtained from adrenal extract to determine their effect on metabolism.

A study has been made of the effects of whole extract, cortin, sodium factor, desoxycorticosterone acetate and sodium salts in adrenalectomized dogs. Their effects on basal metabolism as well as on the S.D.A. of protein, fat and carbohydrate have been followed.

METHODS

Care of animals. Adult female dogs weighing 5 to 10 kg. were selected from a colony which had been kept in our laboratory for many weeks in order to be sure that they were in good condition and that they thrived under laboratory conditions. They were freed from worms and immunized against distemper. Their food consisted of a mixture of ground Purina Dog Chow and beef heart. The adrenalectomized animals all received 300 gm. of beef heart and 70 gm. of Purina Dog Chow daily unless otherwise stated. Cod liver oil was administered once a week. Sodium salts were added in some instances as noted in the text. The amount of food was adjusted so that a fairly constant weight was maintained throughout the experimental period. The dogs were fed once each day at 5 P.M. The animal quarters were maintained at a temperature of 24 to 27°C. Adrenalectomy was performed in 2 stages under aseptic conditions. The second operation was followed by treatment with whole adrenal extract until complete recovery, as evidenced by the blood picture (sodium, potassium and urea concentration and cell volume).

Adrenal preparations. The following preparations were used: unfractionated adrenal cortical extract (whole cortical extract) (10), unfractionated whole adrenal extract (whole adrenal extract) (11), cortin fraction (9), sodium factor fraction (9), and desoxycorticosterone acetate.[2] All were in aqueous solution except the sodium factor which was dissolved in 5% propylene glycol and the desoxycorticosterone acetate which was dissolved in peanut oil.

All injections were made subcutaneously twice daily at 8 A.M. and 5 P.M. Sufficient extract was employed to maintain the animal in good condition as determined by normal values for sodium and potassium in the plasma and urea in the blood for all animals except those treated by cortin. In the latter instance the animal was considered in good condition if his blood urea was low and he reacted in a normal manner.

Measurement of the respiratory metabolism. For the metabolic studies the Tissot-Haldane technic, as described by Boothby and Sandiford (12), was used. As a check the respiratory quotient of a small alcohol lamp was determined at intervals during the course of the experiments and the values obtained were compared with the theoretical. The Haldane analyzers were frequently checked by the analysis of outdoor air. In order to connect the animal to the spirometer a special mask was made. This consisted of a metal cone so constructed as to fit the muzzle of each subject fairly closely. It was strapped to the head and made air-tight about the nose and corners of the mouth by means of strips of rubber dental dam, adhesive tape and petrolatum. Repeated tests with soap suds or a mirror revealed no leaks. The metal mask was connected to the usual valves of the Tissot spirometer by means of rubber tubing. Previous to the actual tests the animals were trained to lie quietly connected to the apparatus for periods of 15 minutes or more.

On the morning of an actual test day the animal was carried from the animal quarters to the metabolism room and allowed a rest of at least one hour, after which she was put on a comfortable bed and connected to the apparatus with as little

[2] Desoxycorticosterone acetate, in the form of Doca, was supplied by Roche-Organon, Inc., Nutley N. J., through the courtesy of Dr. R. D. Shaner.

manipulation as possible. All basal tests were run between 10 A.M. and 12 M., that is 17 to 19 hours after the last feeding. Usually two 7- to 10-minute basal tests were made and if they agreed closely they were averaged; if not, the lower one was taken as that day's basal. Any test during which there was a major movement on the part of the animal was discarded. The tests were made at an environmental temperature of 25 to 28°C. If the specific dynamic action of a particular foodstuff was to be studied, this was fed following the basal tests. The amount of the food used in an experiment was determined primarily by the quantity the dogs would readily take and retain. Ground beef heart was the protein test food. The maximum amount which a dog would consume completely in 5 minutes was 100 gm. containing 16 gm. of protein. Cream containing 32% fat was given by a stomach tube. The maximum amount which could be retained at one time was 150 cc. This would contain 48 gm. of fat, 4.1 gm. of protein, and 6 gm. of carbohydrate. Considerable difficulty was encountered in giving the dextrose used as the carbohydrate food. To the normal dogs, 40 gm. dissolved in 120 cc. of distilled water was given by stomach tube. After adrenalectomy, even with apparently adequate treatment, some animals were unable to retain this amount so it had to be cut to 25 gm. in later experiments. In another series of experiments the effect of 15 gm. of dextrose in 120 cc. of distilled water given orally, was compared with the same amount of dextrose given intravenously in 50 cc. of physiological saline. This particular quantity of glucose was chosen since it was found to be the largest amount which could be injected over a 5-minute period without large losses through the kidney. With this dose the kidney excreted 1.5 to 2.5 gm. during the 5-hour experimental period. Experiments in which the saline alone was administered intravenously were used as controls. All foods were warmed to approximately 37°C. before ingestion.

After the food had been given, 7- to 10-minute metabolism periods were run at intervals during a 4-hour period in the case of protein, a 5-hour period in the case of glucose, and a 9.5-hour period in the case of fat. A series of metabolic tests on carbohydrate or protein consisted of 4 or 5 periods so spaced by trial and error in the normal animal as to fall one on the upward trend of the calorigenic action of the particular food, one at or near the peak, and two on the downward sweep of the curve. In the case of the 15 gm. portion of dextrose, even when it was given orally, the S.D.A. occurred so rapidly that no period was obtained during the increasing phase. For fat, since the curve is of much longer duration, 6 tests were run after food, omitting the usual two basals. Two were distributed on the upward trend, one at or near the peak and one on the downward trend. This could not be done for the treated adrenalectomized dogs because such procedure would extend the experiment beyond a feasible time limit. However the period of observation was long enough to show a change from the normal.

All metabolism data were calculated on the basis of cal./sq.m./hr. using the simplified formula of Cowgill and Drabkin (13) for surface area.

Blood analysis. Blood for sugar determinations was taken from a marginal ear vein by means of a needle prick which did not disturb the animal. The determinations were made by the Shaffer-Hartman method as modified by Somogyi (14). The blood sugar curve was followed in both normal and treated adrenalectomized dogs for periods of 3.5 to 6.0 hours after the oral or intravenous administration of 15, 25 and 40 gm. portions of dextrose.

Methods for the determinations of electrolytes have been described elsewhere (15). Urea nitrogen was determined by the method of Van Slyke (16). The volume per cent of packed erythrocytes was determined by the method of Wintrobe (17).

RESULTS

Basal metabolism. Basal metabolism was studied in 4 normal dogs and in 3 adrenalectomized dogs treated with different adrenal preparations. In addition, one of the adrenalectomized animals was studied while being maintained on sodium salts alone and another on desoxycorticosterone acetate. No periods were run earlier than the fourth day on any regimen.

Three of the normal dogs showed basal metabolic rates as follows: in *dog B* the average of 8 tests was 32.60 cal./sq.m./hr. with a standard error ($\sigma/\sqrt{n}$) of 0.50; in *dog H*, an average of 6 tests gave 37.89 cal./sq.m./hr., with a standard error of 0.62;

TABLE 1. BASAL METABOLISM OF *DOG B1* (8 kg.)

Normal			*Adrenalectomized Treated with*								
			Whole cortical extract[1]			Cortin			Na factor		
Date 1938	cal./ sq.m./hr.	R.Q.	Date 1939	cal./ sq.m./hr.	R.Q.	Date 1940	cal./ sq.m./hr.	R.Q.	Date 1940	cal./ sq.m./hr.	R.Q.
10/18	35.54	0.701	3/14	29.35	0.724	3/7	37.19	0.788	4/16	35.62	0.774
10/21	37.25	0.727		26.71	0.700		37.65	0.774	4/18	34.73	0.727
	35.50	0.713	3/22	28.80	0.739	3/12	35.31	0.742		35.58	0.716
10/25	33.55	0.732	4/13	29.99	0.683	2/13	30.61	0.763	4/22	32.07	0.773
	32.73	0.767	4/18	28.00	0.703	2/21	31.86	0.728	4/24	34.75	0.773
11/3	34.03	0.719		31.22	0.718		34.30	0.788		36.52	0.747
12/22	32.49	0.755	6/6	30.28	0.705		34.21	0.826	Av.	34.88	0.751
12/28	33.81	0.700		25.70	0.788	3/20	33.67	0.787			
Av.	34.36	0.727	Av.	28.75	0.720	Av.	34.35	0.774			

[1] Wt.—6.7 kg.

and in *dog G* an average of 6 tests gave 37.85 cal./sq.m./hr. with a standard error of 0.62.

Dog B1 whose average normal basal metabolism was 34.36 cal./sq.m./hr. showed a drop in metabolism to 28.75 cal./sq.m./hr. after adrenalectomy when treated with whole cortical extract. She appeared to be in excellent condition. It seems likely that the lowered basal rate in this animal bore no relation to the treatment but was, rather, the result of her undernourished condition at this time. Before the second operation her weight was 8.6 kg. At the time of these tests her weight was 6.7 kg., a reduction of 22%, while the metabolism was reduced 16.3%. The plasma sodium, potassium and blood urea were normal but the cell volume was low. The actual values before adrenalectomy were: sodium 143.2 m.Eq./l.; potassium 4.48 m.Eq./l.; blood urea nitrogen 16 mg. %; erythrocytes 59 vol. %. After adrenalectomy when maintained on whole cortical extract the values were: sodium 147.5 m.Eq./l.; potassium 4.3 m.Eq./l.; blood urea nitrogen, 18 mg. %; erythrocytes 42 vol. %. Her loss of weight and low erythrocyte volume may be explained by hemorrhage which occurred due to an accident at the time of the second operation 2 months previously. Later she had a normal metabolic rate when treated with either cortin alone or sodium factor alone (table 1). By this time her weight had returned to normal. Two other dogs (19 and 20) whose metabolic rates were not determined before adrenalectomy showed the same metabolism when maintained on unfractionated extract, cortin or sodium factor separately and the values lay within the range of the normal animals (table 2, 3).

Dog 20 was also maintained on sodium salts. Sodium salts were given daily as follows: 6 gm. sodium chloride, 1 gm. sodium bicarbonate, 0.5 gm. sodium citrate containing a total of 118.2 m.Eq. of sodium. Her metabolism was unchanged.

It seemed that any treatment which maintained the animal in good condition as determined by appetite, behavior and blood urea supported a normal basal metabolic rate. This was true for cortin, sodium factor, desoxycorticosterone acetate and sodium salts as well as for whole adrenal extract.

Specific dynamic action. Protein. The S.D.A. of protein was studied in one dog (*B1*) before adrenalectomy and afterward when being maintained on whole cortical extract, on cortin alone and on sodium factor alone (fig. 1). Each block represents the average of two or three tests. The figures for individual tests are not given since they showed similar trends. In the normal animal, it will be noted that the greatest per-

TABLE 2. BASAL METABOLISM OF *Dog* 19 (8 KG.) AFTER ADRENALECTOMY

Whole Adrenal extract			Cortin			Na factor			Desoxycorticosterone acetate		
Date 1939	cal./ sq.m./hr.	R.Q.	Date 1939	cal./ sq.m./hr.	R.Q.	Date 1939	cal./ sq.m./hr.	R.Q.	Date 1940	cal./ sq.m./hr.	R.Q.
9/19	29.53	0.729	11/29	30.38	0.816	12/8	33.66	0.706	3/22	33.39	0.699
	32.76	0.718		34.68	0.769		31.70	0.677		32.89	0.689
9/27	29.04	0.797	12/1	34.07	0.794	12/11	30.23	0.721	4/15	36.00	0.705
9/29	32.66	0.720		34.54	0.750	12/19	35.73	0.707	4/17	32.09	0.704
	36.52	0.722	12/5	35.00	0.767	12/27	36.08	0.721	Av.	33.59	0.699
10/4	34.69	0.716		32.47	0.743	Av.	33.48	0.706			
	35.64	0.720	12/6	35.59	0.826						
10/7	30.32	0.805		34.97	0.793						
	30.54	0.753	Av.	33.96	0.782						
Av.	32.41	0.749									

TABLE 3. BASAL METABOLISM OF *Dog* 20 (10 KG.) AFTER ADRENALECTOMY

Whole adrenal extract			Cortin			Na factor			Na salts		
Date 1939	cal./ sq.m./hr.	R.Q.	Date 1938	cal./ sq.m./hr.	R.Q.	Date 1939	cal./ sq.m./hr.	R.Q.	Date 1939	cal./ sq.m./hr.	R.Q.
3/23	32.79	0.769	9/26	34.52	0.739	1/18	35.92	0.699	2/13	36.71	0.724
	34.68	0.768	9/28	36.87	0.777		36.96	0.711		36.66	0.726
Av.	33.74	0.769		34.27	0.787	1/21	36.64	0.695	2/22	32.52	0.682
			10/5	38.44	0.787		35.79	0.723	2/27	35.37	0.707
				35.66	0.778	Av.	36.33	0.707	3/8	33.36	0.717
			10/11	36.67	0.773					34.91	0.689
				38.30	0.789				Av.	34.92	0.708
			Av.	36.39	0.776						

centage increase occurred 45 minutes after the ingestion of the protein, an increase of 9.70 cal./sq.m./hr. or 35.8% over the basal and at 3 hours and 40 minutes it had already declined to 1.50 cal./sq.m./hr. or within 4.3% of the basal level. Compare to this, the blocks representing the metabolism of the adrenalectomized animal under treatment with the different extracts. In all cases the high point in the S.D.A. curve was moved to the 2-hour period, this point representing for whole extract treatment a 41.5% increase, for cortin a 31.8% increase and for sodium factor a 26.4% increase over the basal level. At the end of 3 hours and 40 minutes the metabolism of the adrenalectomized animal under treatment was still 10.8% for whole extract, 20.2% for cortin and 20.7% for sodium factor as compared to only 4.3% above the basal level in the normal animal. There was, therefore, in all cases, a significant decrease in the rate of development of the S.D.A. of protein even with this small test meal.

Carbohydrate. The S.D.A. of 40 gm. of dextrose was studied on *dog B1* before adrenalectomy and afterward while being maintained on unfractionated adrenal extract or on cortin alone (fig. 2). The data on sodium factor treatment represented in

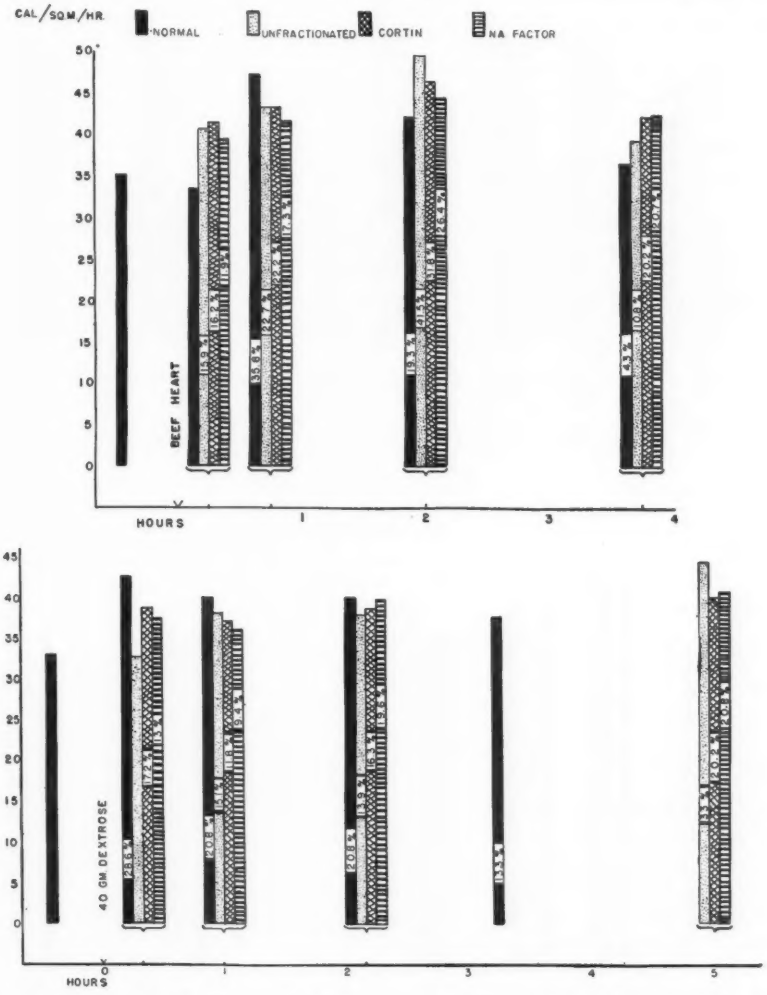
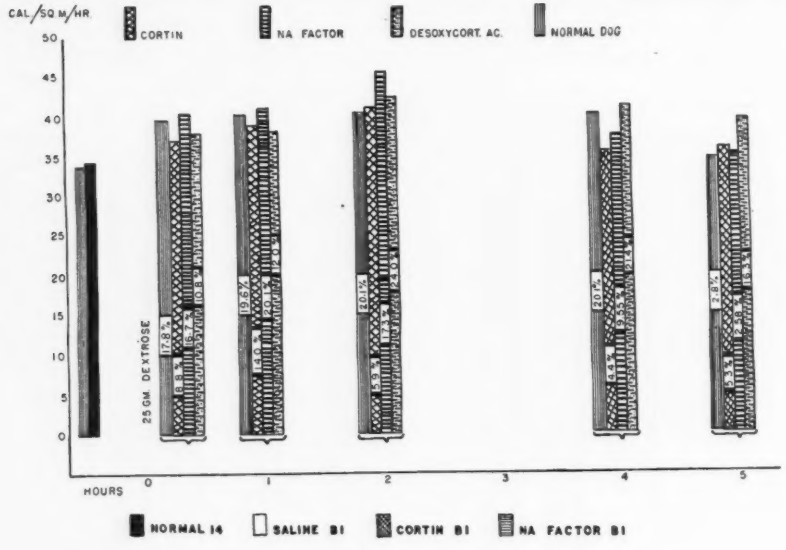


Fig. 1 (above). S.D.A. OF PROTEIN IN *dog B1* BEFORE ADRENALECTOMY (solid black) AND AFTERWARD WHEN TREATED WITH UNFRACTIONATED CORTICAL EXTRACT, CORTIN AND Na FACTOR. Fig. 2. (below). S.D.A. OF 40 GM. DEXTROSE IN *dog B1* BEFORE ADRENALECTOMY (solid black) AND AFTERWARD. Treatment with unfractionated cortical extract and cortin. Data on Na factor taken from *dog 19*.

the chart are taken from work on another adrenalectomized dog (19) of the same weight. In the normal animal the greatest percentage increase occurred 20 minutes after the carbohydrate meal. The metabolism rose 9.5 cal./sq.m./hr. or 28.6% above the basal and at 3 hours and 20 minutes it was only 4.5 cal./sq.m./hr. or 13.3 % above



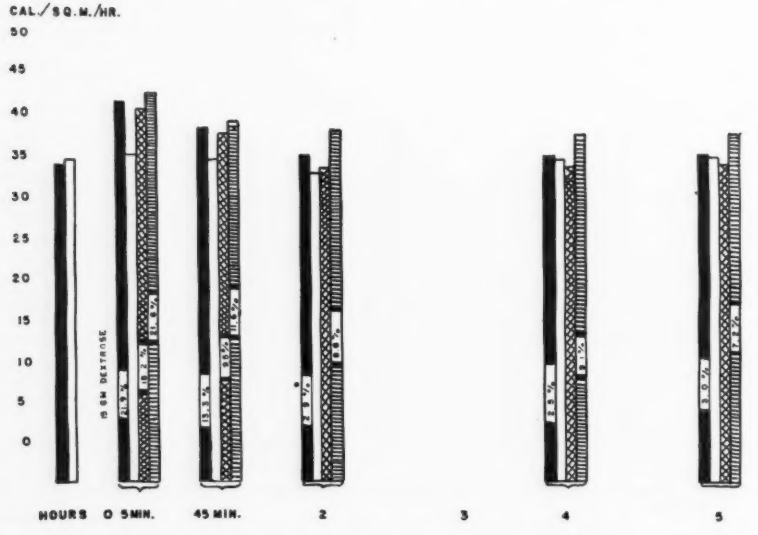


Fig. 3 (above). S.D.A. OF 25 GM. DEXTROSE IN A NORMAL DOG AND *dog B1* AFTER ADRENALECTOMY TREATED WITH CORTIN AND Na FACTOR. Basal of *dog B1* in solid black. Data on desoxycorticosterone acetate taken from *dog 19*. Fig. 4 (below). S.D.A. OF 15 GM. DEXTROSE INTRAVENOUSLY IN *dog 14*, NORMAL AND IN *dog B1* AFTER ADRENALECTOMY. Control data on *dog B1* after an equal volume of physiological saline intravenously.

the basal rate. This was in striking contrast to the S.D.A. in the extract-maintained adrenalectomized animal. Here, in all cases, the high point of metabolism occurred 5 hours instead of 20 minutes after ingestion of the dextrose and in spite of the

lengthening of the experiment by 1 hour and 40 minutes no points were obtained on the downward trend.

However, with 25 gm. of dextrose (fig. 3) it was found that the treated adrenalectomized animals showed an S.D.A. curve more like the normal when given 40 gm. of dextrose. In this series of experiments cortin, sodium factor and desoxycorticosterone acetate were used. The shift to the right was not nearly so marked as with the 40 gm. portion of dextrose given to the treated animal. The high point occurred at the 2-hour instead of the 5-hour period. Five hours after ingestion of the carbohydrate the S.D.A. curves were within 5.3% (cortin), 2.6% (sodium factor) and 16.3% (desoxycorticosterone acetate) of the basal level. It appeared that desoxycorticosterone acetate was not quite so effective in restoring the S.D.A. curve to normal.

The series of experiments in which 15 gm. of dextrose was administered orally to the same adrenalectomized animal (*B*1) showed exactly the same slope with the high point of the curve coming at the second hour; for cortin it was 12.42% increase over basal and for sodium factor it was 13.05%. By the fifth hour the curve for cortin had returned to the basal level while that for sodium factor was within 3.1% of the base line.

Fifteen grams of dextrose administered intravenously to a normal and to an adrenalectomized dog maintained on either cortin or sodium factor produced curves of the same trend (fig. 4). The high point occurred during the first period which was 5 to 15 minutes after the dextrose injection and by the fourth hour the curves for the normal and cortin treated adrenalectomized dogs had returned to the basal value. The same adrenalectomized animal treated with sodium factor showed a supernormal plateau from the second through the fifth hour. It will be seen from these data that under the various kinds of treatment, there was a marked decrease in the rate of development of the S.D.A. of carbohydrate even with as little as 25 gm. or 15 gm. of dextrose administered orally. When the smaller amount of dextrose was administered intravenously this difference in rate entirely disappeared.

Dextrose tolerance curves. As a possible means of determining how great a part decreased rate of absorption might play in the delayed curve of S.D.A., dextrose tolerance curves were studied after intravenous or oral administration of this substance. In the normal dog the high point of the curve came 30 minutes after oral administration of 15 gm. of dextrose (fig. 5). In 3.5 hours the curve had returned to a value within the normal range for this animal.

An adrenalectomized dog (*B*1) on cortin therapy showed the high point in the blood sugar curve (fig. 5) only after an interval of 60 minutes and it was 40% lower than the high point on the normal curve. Furthermore there was a long supernormal plateau in the curves which suggested an abnormally long period of absorption since it did not occur following intravenous glucose administration. The animal was in excellent condition as indicated by blood urea (8 mg.%), appetite and behavior. The sugar tolerance curve, while this same animal was being maintained in good condition on sodium factor (fig. 5), was slightly higher but very similar in trend to that on cortin therapy. This animal treated with desoxycorticosterone acetate showed an extremely flat sugar tolerance curve, though she was receiving 1.5 mg. per day, a dose which later proved to be 3 times her minimum maintenance level. Larger amounts of dextrose, namely 25 gm. and 40 gm. doses, given to adequately treated adrenalectomized dogs, showed similar curves except that the plateaus occurred at higher blood sugar levels as would be expected. On the other hand when 15 gm. of dextrose was administered intravenously to *B*1 there was no deviation from the normal with any therapy (fig. 5). It would appear, therefore, that in the case of cortin, sodium factor

and desoxycorticosterone acetate, at least, absorption is a large, if not the sole factor in shifting the high point in the S.D.A. of carbohydrate to the right.

Fat. The S.D.A. of fat was studied in 2 dogs. In dog B1 determinations were made before adrenalectomy and afterward while she was being carried on unfractionated cortical extract, cortin and sodium factor (fig. 6). It will be seen that the high point in

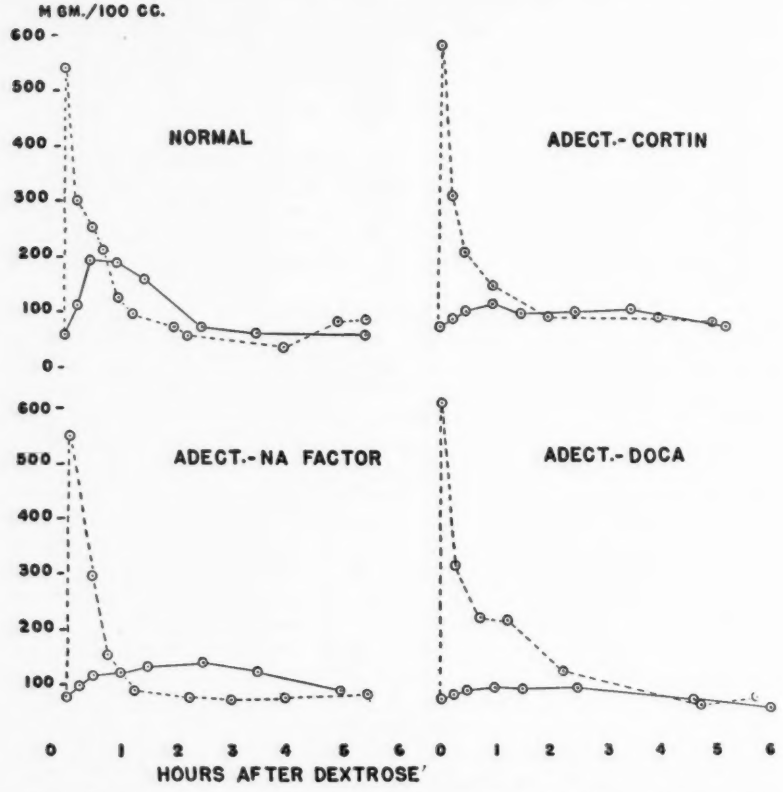


Fig. 5. Blood sugar curves following administration of 15 gm. dextrose in a normal dog and in a treated adrenalectomized dog. Oral, continuous line; intravenous, broken line.

the S.D.A. of fat for the normal animal occurred 1 hour after the ingestion of the cream with a well maintained plateau through the third hour. By the end of 9.5 hours the metabolism had returned to the basal level. In other words the whole S.D.A. of 48 gm. of butter fat occurred within a 9.5-hour period. Compare to this the S.D.A. of fat occurring in the treated-adrenalectomized animal. In all cases the high point in the curve was shifted far to the right. For unfractionated cortical extract and sodium factor it occurred at the 9.5-hour period while for cortin it appeared at 7 hours. At the 9.5-hour period the curves for unfractionated cortical extract, cortin and sodium factor were 21.6%, 21.2% and 40.1% respectively above the basal level.

Desoxycorticosterone acetate was employed in a second adrenalectomized dog

(19) of the same weight. The curve of the S.D.A. of 48 gm. of fat in this animal was very similar to that of *dog B1* on cortin therapy.

It is apparent that only a portion of the S.D.A. of 48 gm. of fat occurred with the 9.5-hour period.

DISCUSSION

Basal metabolism. The values for basal metabolism of dogs reported in this paper agree fairly well with those found in the literature. Thus Lusk and DuBois (18) using Meeh's formula, found the average metabolic rate of 11 female dogs to be 32 cal./sq.m./hr. These data like the results reported in the present work, were obtained under strictly basal conditions as we now define them.

Cowgill and Drabkin (13) in the paper in which they reported the surface area

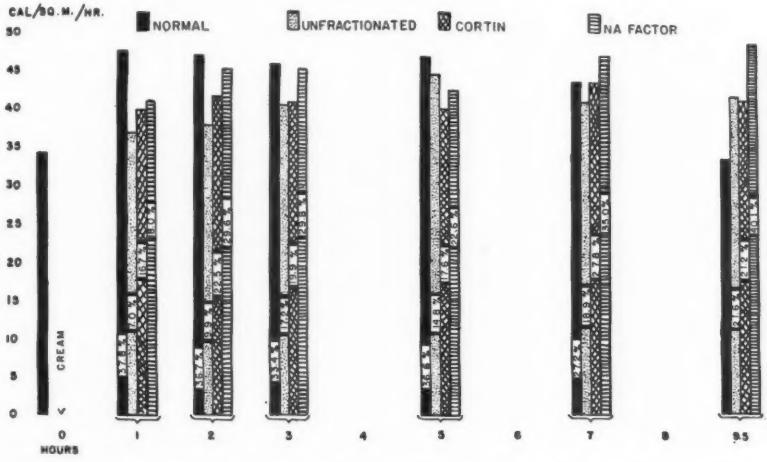


Fig. 6. S.D.A. OF FAT IN *dog B1* BEFORE ADRENALECTOMY AND AFTERWARD WHEN BEING TREATED WITH UNFRACTIONATED CORTICAL EXTRACT, CORTIN AND Na FACTOR.

formula used here, determined the average basal metabolic rate for one female dog over a period of 4 years and recalculated some data from Lusk on another female dog obtained over a period of months. Their results, 36.61 and 33.71 cal./sq.m./hr. respectively, agree well with those reported in this work.

Harrop and coworkers (19) reported detailed protocols on 2 male dogs studied while in "good condition" on unfractionated cortical extract. One animal was maintained on extract for too short a period, i.e. 6 days after operation, to allow a return to the normal metabolic rate. In the other dog (*P-2*) a 6-week period was allowed after operation, for complete recovery. This animal when treated with extract showed an oxygen consumption of 56.1 cc./min. as compared with an average of 53.4 cc./min. for the normal controls. In the present work, typical experiments on *B1* showed a basal oxygen consumption for the normal, 43.43 cc./min.; for unfractionated cortical extract treated, 35.41 cc./min.; for cortin treated 41.23 cc./min., and for sodium factor treated 42.35 cc./min. These agree fairly closely among themselves except in the value for the cortical extract, which is explained in the section on results. They are somewhat lower than those found by Harrop, first, because these dogs were females and his were males and second, probably, because he maintained "basal

conditions" for a period of 45 to 60 minutes. It was the experience in this work that strictly basal conditions could not be maintained for so long a period in the dog.

Thorn and coworkers (5) stated that they found no change in the fasting R.Q. in adrenalectomized dogs implanted with desoxycorticosterone acetate pellets. In the dog on desoxycorticosterone acetate injections, used in these experiments, the R.Q. was 0.02 lower than the average of all the normal dogs but about 0.02 higher than the average for one of the 4 normal dogs. The differences are insignificant. These experiments would, therefore, offer confirmation of Thorn's results.

Specific dynamic action. It will be noted (fig. 1) that *B*1 showed an abnormally high S.D.A. on the protein meal, when she was treated with unfractionated cortical extract. This is because of her state of undernutrition and low basal rate which has already been discussed under results. It has been shown by Mason (20), for emaciated human subjects with low basal rates, that the S.D.A. of a standard protein meal is much greater than for normal persons. In fact it raises their metabolism to the level it would have reached if the subjects' basal rate had been normal. In the case of dog *B*1 the metabolism rose somewhat above the normal figure.

It has been found by Lips (21) and confirmed by Schaeffer and coworkers (22) that splanchnicotomy in the dog considerably decreased the S.D.A. of protein. They thought that the disturbance was caused by cutting the nervous pathways to the adrenal medulla. Murlin (23) in reviewing the subject aptly commented that the observed effects might be due to decreased absorption caused by the operation. Until this is ruled out the adrenal medulla as a causative factor in the S.D.A. of protein must remain in doubt. Since, in the present work, no actual lowering of the S.D.A. of the foodstuffs but only a delayed development was observed it was deemed improbable that the absence of the adrenal medulla was an important factor.

A few words might be said concerning the light that these experiments, coupled with others, might throw on the intermediary metabolism of amino acids in adrenalectomized treated animals. Since Nord and Deuel (7), even in untreated adrenalectomized dogs, found no significant difference between the S.D.A. of 10 gm. of glycine administered orally or intravenously it seems unlikely that the difficulty which altered the S.D.A. curve reported in this work, was one of absorption of the amino acids. Nord and Deuel found that adrenalectomized dogs produced an average of 2.99 extra cal./hr. when the glycine was given orally and 2.87 extra cal./hr. when it was administered intravenously.

The difficulty might conceivably lie in a decrease in the speed of breakdown of the protein in the gastrointestinal tract, to form amino acids. This has not been tested. However, from work done on the intermediary metabolism of carbohydrate after adrenalectomy in both treated and untreated animals (discussed under S.D.A. of dextrose) it would appear that liver function, at least in so far as its ability to convert lactic acid to glycogen and to store glycogen is concerned, is markedly disturbed. Relatively large quantities of extract, far in excess of the ordinarily accepted maintenance level, were required to restore liver function to normal. Since deaminization is known to take place chiefly in three organs, i.e. the liver, kidney and intestinal mucosa and since Lundsgaard (24) has quite conclusively demonstrated that by far the major portion of the S.D.A. of amino acids is associated with their deaminization, it would seem probable that a decrease in the rate of development of the S.D.A. of protein, such as has been observed in the present experiments, might be traced to impaired function of these organs. Furthermore, it has been shown by Jimenez-Diaz (25) that the rate of deaminization of amino acids is decreased in kidney slices from adrenalectomized animals. Russel and Wilhelmi (26) have demonstrated that as in the case of liver function, relatively large amounts of adrenal extract or desoxycorticosterone acetate were required to restore the deaminization rate to normal.

Carbohydrate. The blood sugar curves after oral and intravenous administration of dextrose indicated that the delayed development of the S.D.A. of carbohydrate in the cortin, sodium factor and desoxycorticosterone acetate series, at least, might be due to delayed absorption. The higher plateau of blood sugar values with 40 gm. rather than with 25 gm. of dextrose given orally might account for the abnormally sustained S.D.A. curve in the former case. Cori and Cori (27) observed a decrease in the rate of absorption of glucose from the intestine of adrenalectomized rats. Wilbrandt and Lengyel (28) showed that 2 to 4 cc. of adrenal extract injected twice daily into adult rats restored the absorption rate to normal. Thorn et al. (6) found that it required 30 to 50 cc. (180–300 cat units) of adrenal extract to return the carbohydrate metabolism of an Addison's disease patient to normal. We used, daily, 2 cc. of adrenal extract of about the same potency per cc. as that used by Thorn. This was apparently inadequate to maintain the carbohydrate metabolism.

Desoxycorticosterone acetate produced much less improvement of the tolerance curve of orally administered glucose in the dog (fig. 5) than it did in the patients with Addison's disease just cited, probably because more material was available from the implanted pellets which Thorn used, than from the oil injections which we employed.

Though there is no doubt that absorption plays a role in delaying the rise of the S.D.A. curve, another factor might also have been operating, namely a disturbance in the rate of conversion of dextrose to glycogen. Baur (29), and Dann and Chambers (30) have shown that the S.D.A. of glucose is due primarily to its conversion to glycogen though not necessarily in the liver since Wilhelmj (31) and coworkers observed that the S.D.A. of glucose might even be increased after hepatectomy. The fact that the S.D.A. of carbohydrate is concerned with its conversion to glycogen might be of significance in explaining the delayed curve in adrenalectomized, treated animals. Many years ago it was observed (32, 33) that liver glycogen was decreased to the vanishing point in adrenalectomized dogs and monkeys surviving the operation several days. More recently it has been shown (34, 35) that large quantities of extract representing 400 to 1600 gm. of adrenal tissue will restore the liver glycogen of adrenalectomized cats and rats to normal or above. This dosage is far above the maintenance level for electrolytes. Furthermore, Buell, Anderson and Strauss (36) observed that adrenalectomized rats treated with sodium chloride had low liver glycogen values. It is, therefore, quite probable that a slower rate of conversion of glucose to liver glycogen played a part in delaying the S.D.A. curve of carbohydrate.

Fat. The S.D.A. of fat as found by Lusk (37) was 4.1% of the caloric content of the fat ingested, while in our work it represented 6.3% of the heat value of the fat administered. This rather large discrepancy was due, in all probability, to the effect of the other nutrients in the cream. The 150 cc. portion contained, in addition to the fat, 4.1 gm. protein and 6.0 gm. of carbohydrate.

It will be noted that the normal S.D.A. curve of fat (fig. 6) rose to its high point at the first hour with a fairly well sustained plateau over a 5-hour period. Murlin and Lusk (38), using "75 gm. of emulsified fat" reported their peak of S.D.A. at the sixth hour. However, the two curves agree in that both returned to the base line in about 9.5 hours. The cream might have been more readily absorbed than the "emulsified fat" which consisted of peanut and lard oils.

SUMMARY

Any treatment such as cortin, sodium factor, desoxycorticosterone acetate or sodium salts, which maintained an adrenalectomized dog in good condition, supported a normal basal metabolic rate. Adrenalectomized dogs kept in good condition with unfractionated adrenal extract, cortin or sodium factor showed a significant delay in the rate of development of the S.D.A. of protein. Such animals showed a more marked

delay in the rate of development of the S.D.A. of carbohydrate when 40 gm. of dextrose was administered while with 25 gm. or 15 gm. the rate approached normal. Desoxycorticosterone acetate was less potent than other adrenal preparations in restoring the S.D.A. of carbohydrate. Sugar tolerance curves determined after oral and intravenous administration of as small an amount as 15 gm. dextrose, to a cortin, sodium factor or desoxycorticosterone acetate treated adrenalectomized dog, showed delayed absorption. These curves indicated that a slower rate of absorption played an important part in the retarded development of the S.D.A. of carbohydrate. In the case of fat, also, there was a marked delay in the rate of development of the S.D.A. in these treated adrenalectomized animals.

We wish to thank Dr. Oliver Kamm of Parke, Davis and Company for adrenal glands.

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REVERSAL OF FATIGUE IN THE ADRENALECTOMIZED RAT BY GLUCOSE AND OTHER AGENTS[1]

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THE RAT loses its ability to perform a normal amount of work within a few hours after its adrenal cortices are removed (1). The ability to work can be partially restored by treatment with certain of the secretory products of the adrenal cortex (2, 3). The purpose of this study was to investigate the reversibility of the fatigue of the adrenalectomized rat under certain conditions by treatment with substances regulated by adrenal cortical hormones and especially to compare the efficacy of intravenous infusions of glucose, sucrose and sodium chloride solutions at equivalent isomolar concentrations. The results emphasize the apparently greater importance of changes in carbohydrate metabolism rather than in electrolyte metabolism in so far as the ability of the adrenalectomized rat to perform muscular work is concerned under the conditions of these experiments.

METHODS

The animals used in these experiments were male rats of the Sprague-Dawley strain having a body weight of 180 ± 2 gm. Purina Dog Chow was used as a maintenance diet.

Faradic stimulation of the muscle was applied by the method described by Heron, Hales and Ingle (4). The frequency of stimulation was controlled by a contactor driven by a small telechron motor which closed the circuit for a period of approximately 10 sigma 3 times each second. The contractions of the muscle were transmitted to the pulley of an automatic device which recorded the distance the muscle shortened at each contraction. This recorder was a one-way recording Veeder counter to which was attached a compact clutch built within the pulley of the counter. The shortening of the muscle was recorded, its relaxation was not. Each recorder revolution represents the lifting of a 100 gm. weight the distance of 4 cm. or approximately 400 gm.-cm. of work.

Completely adrenalectomized animals were subjected to the work test immediately following operation. Adrenal demedullation was performed on rats weighing 45–70 gm. and normal rats of the same weight range were subjected to sham operations. All of the adrenal demedullated and sham-operated rats were maintained until they reached a body-weight of 180 gm. and were then subjected to the work test.

The animals were anesthetized with phenobarbital sodium administered subcutaneously. The animals were then mounted upon an animal board which would accommodate 6 rats. The distal end of the left tibia was exposed and secured in a vertical position by a hemostat. A silver needle was inserted between the gastroc-

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nemius and biceps muscle exactly on a horizontal level with the knee joint. A 100 gm. weight was attached by a strong linen thread to the freed tendon of the gastrocnemius, and looped about the pulley of the recorder. The hemostat and needle served as electrodes. The electrodes of different animals were connected in series.

The infusions were made in the femoral vein of the right hind leg with a 25-gauge needle. The time for the making of infusions was 90 ± 10 seconds. Records of the rate of work were made for one minute at 5-minute intervals following the beginning of injection, and are expressed as the number of recorder revolutions per minute.

TABLE 1. DIFFERENCES BETWEEN MEANS AND THE RELIABILITY OF THESE DIFFERENCES

Experiment	Groups compared	Diff. of means	Sum of diffs. of means	Sigma diff.	t
3	3.0 cc. Glucose 3.0 cc. NaCl		18.2	2.04	8.9
	3.0 cc. Glucose 3.0 cc. Sucrose		16.1	2.21	7.3
	3.0 cc. Sucrose 3.0 cc. NaCl		2.1	1.33	1.6
4	0.3 cc. Glucose 0.3 cc. NaCl	3.4		0.40	8.5
	3.0 cc. NaCl 0.3 cc. NaCl	2.8		0.33	8.5
	3.0 cc. Glucose 0.3 cc. Glucose	2.4		0.45	5.2
5	3.0 cc. Glucose 3.0 cc. Glucose and NaCl	0.6		0.62	0.97
6	Adrenalin chloride Adrenalin chloride and 3.0 cc. NaCl	0.8		0.66	1.2
	Adrenalin chloride Adrenalin chloride and 3.0 cc. glucose	5.8		0.87	6.5

Values represent number of recorder revolutions per minute.

Means and distribution of values are shown in the accompanying graphs.

From *experiment* 3, the difference in mean responses for each osmotically equal concentration of NaCl, glucose and sucrose was calculated and the sum of these mean differences is expressed in the above table.

Values for t of 2.0 or greater indicate that the difference between means is statistically significant. Values less than 2.0 indicate that either a true difference does not exist between the means or that it is small.

In the analysis of the data averages were calculated as arithmetical means. The variances of the differences between means are expressed in terms of the standard error calculated as described by Fisher (5). In tabulating the differences from the data of *experiment* 3 in table 1, the difference in mean responses for each isomolar concentration of the 3 therapeutic agents was calculated and the sum of these mean differences for each pair of substances is given in the table with the standard error. The calculation of the standard error of the sum of the differences between two series of means is based on the assumption that the variance of the sum is the sum of the variances (6). The values for Fisher's t represent ratios between the difference and the standard error of the difference.

EXPERIMENTS AND RESULTS

Experiment 1. Five normal rats and 5 adrenal demedullated rats were made to work for a period of 10 hours in order to establish standards for the work performance of rats having an intact adrenal cortex with and without the adrenal medulla.

It was found (fig. 1) that the initial rates of work are practically identical for normal and adrenal demedullated rats, but the work output of adrenal demedullated rats falls more rapidly than for normal rats during the first 10 hours of stimulation. This confirms an earlier study (7). Although this early difference is marked it dis-

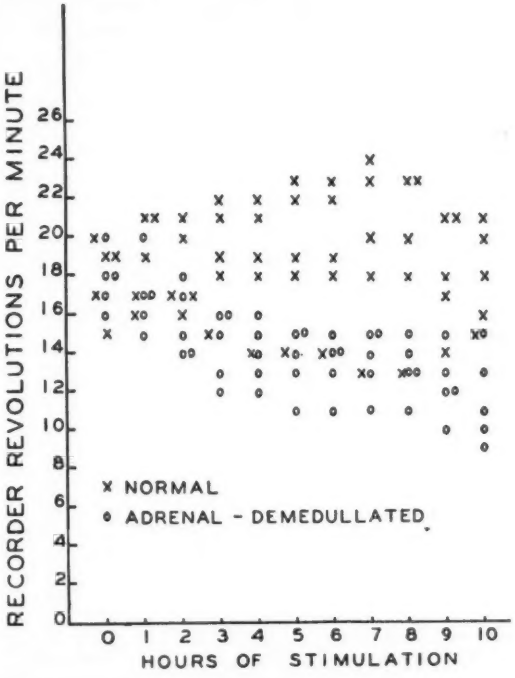

Fig. 1. Work performance of normal and adrenal demedullated rats during the first 10 hours of stimulation. (The low normal values all occurred in one animal.)

appears again upon continued stimulation so that the adrenal demedullated rat is capable of performing as much total work as is the normal rat (7, 8). In these experiments the work curves of normal and adrenal demedullated rats provide standards for estimating the degree of recovery of the adrenalectomized rats which received treatment. In the experiments which follow the basal rate of work was reached and the infusions were made within a period of 4 to 11 hours following the beginning of stimulation.

Experiment 2. The purpose of this experiment was to select a convenient basal rate of work and a standard volume of solution to use for the injection. It was desirable to select a basal rate of work which would represent a very poor performance but which could be depressed to a still lower level by the administration of substances having an unfavorable effect on work. Twenty-two adrenalectomized rats were made to work until the rate was depressed

to the arbitrary level of 3 recorder revolutions per minute. The solution used for infusion was 0.85% sodium chloride. Four rats received 1 cc. each, 4 rats received 2 cc. each, 10 rats received 3 cc. each and 4 rats received 4 cc. each. The volume of 3 cc. gave an optimal increase in the rate of work and was selected as a standard volume. Forty adrenalectomized rats were made to work until the number of recorder revolutions per minute had decreased to 10, 9, 8, 7, 6, 5, 4, 3, 2 and 1 in 4 rats, at each rate of work. Intravenous infusions of 3 cc. of 0.85% saline were given to each animal and the maximum rate of work attained subsequently was noted. The increase in work confirmed the advisability of using 3 recorder revolutions per minute as the basal rate. These data are not summarized graphically.

Experiment 3. This experiment consisted in a comparison of the efficacy of infusions made with 5 different isotonic concentrations of glucose, sucrose and sodium chloride as follows. A total of 90 adrenalectomized rats received intravenous infusions of 3 cc. of a solution at the basal rate of work. Five rats received 0.4% saline, 5 received 2.5 % glucose, and 5 received 5% sucrose. Ten rats received 0.85% sodium chloride, 10 received 5.0% glucose, and 10 received 10% sucrose. Five rats received 1.7% sodium chloride, 5 received 10% glucose, and 5 received 20% sucrose. Five rats received 2.55% sodium chloride, 5 received 15% glucose, and 5 received 30% sucrose. Five rats received 3.4% sodium chloride, 5 received 20% glucose and 5 received 40% sucrose.

All of the animals (fig. 2, table 1) showed increases over the basal rate of work. In each experimental group the recoveries of the animals treated with glucose were significantly more marked than in the animals treated with either sucrose or sodium

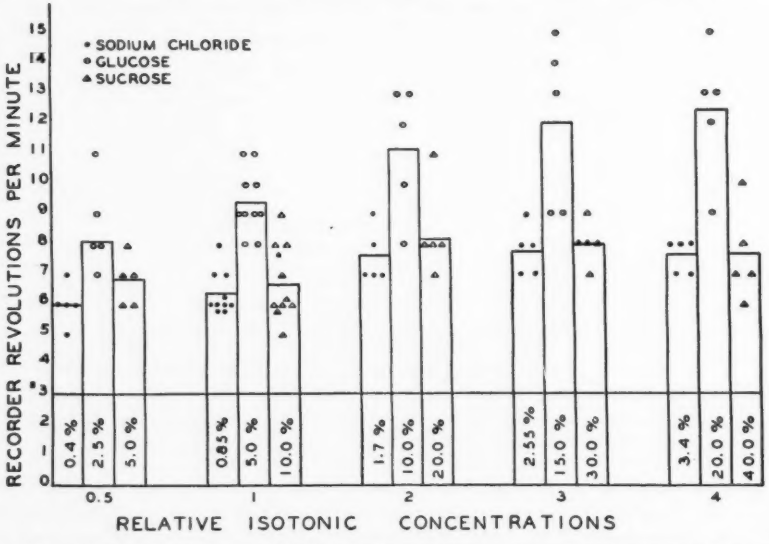

Fig. 2. Effect of solutions of NaCl, glucose and sucrose at different concentrations on the maximum rate of work attained following intravenous infusion (3 recorder revolutions per minute was the rate of work at the time infusions were made, volume of each infusion was 3 cc.).

chloride solutions. In 4 of the 5 experimental groups the average increase of those animals treated with sucrose solutions was only slightly greater than for the animals treated with sodium chloride solution. There was a tendency for the higher concentrations of each of these 3 substances to induce the greatest improvement in the rate of work. The work performance of those animals which were treated with infusions of 15% and with 20% glucose was improved so that their values for work

fell within the range of performance shown by adrenal demedullated rats (fig. 1) at comparable periods of time after the beginning of stimulation. The improved performance of those animals which were treated with glucose was maintained over a slightly longer period of time than that of the animals treated with sodium chloride or with sucrose. For example, the animals which were treated with either 0.85% sodium chloride or with 10% sucrose reached their maximum improvement within the

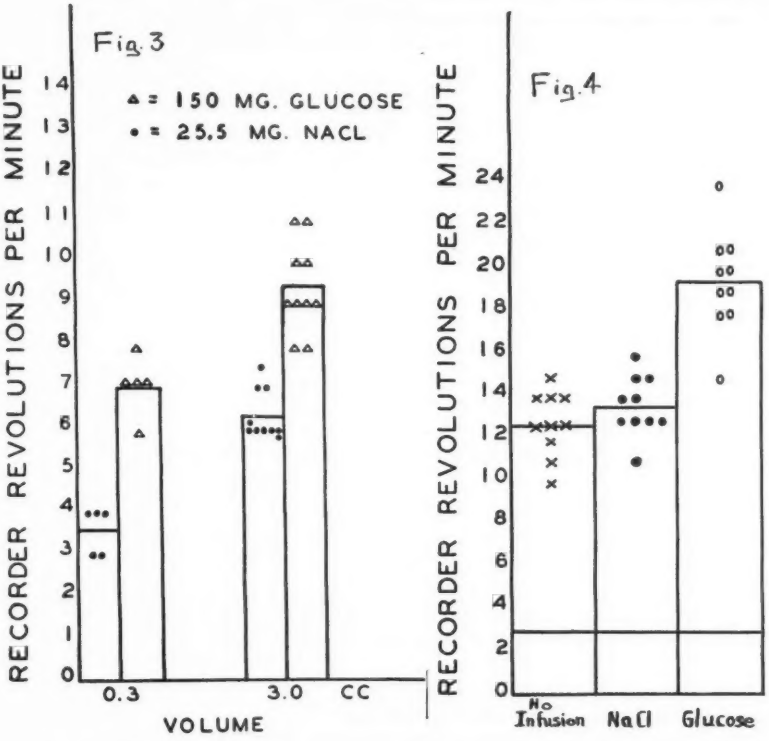

Fig. 3. Effect of volume on the maximum rate of work following intravenous infusion. Fig. 4. Effect of 0.4 cc. of 1:5000 adrenalin given in addition to infusions with 3 cc. of 15% glucose and with 0.3 cc. of 2.55% NaCl on the maximum rate of work attained following treatment.

first 5 to 10 minutes and began to show a secondary decrease in the rate of work within 15 minutes following the beginning of the infusion, whereas those animals which were treated with 5.0% glucose reached their maximum improvement within the first 10 to 20 minutes and began to show a secondary loss in rate of work within 20 to 30 minutes following the beginning of infusion.

Experiment 4. This experiment was proposed to compare the effect on work of injecting very small volumes of osmotically equivalent solutions of glucose and of sodium chloride. Five adrenalectomized rats received infusions of 0.3 cc. each of 50% glucose and 5 adrenalectomized rats received infusions of 0.3 cc. each of 8.5% sodium chloride.

The results (fig. 3, table 1) demonstrated that infusions with a small volume (0.3

cc.) of a 50% glucose solution were significantly more effective in increasing the ability to work than were infusions with 0.3 cc. of 8.5 % sodium chloride. In order to emphasize the volume effect of the infusion the data showing responses to infusions with 3 cc. of 5% glucose and 3 cc. of 0.85% sodium chloride from *experiment* 3 (fig. 2) is included for comparative purposes in figure 3.

Experiment 5. In this experiment 10 adrenalectomized rats received 3 cc. of 15% glucose and 10 rats received 3 cc. of a solution containing 15% glucose and 0.85% sodium chloride. The average maximum rates of work attained (table 1) following intravenous infusion with 15% glucose and with 15% glucose plus 0.85% sodium chloride were 11.2 and 11.8 recorder

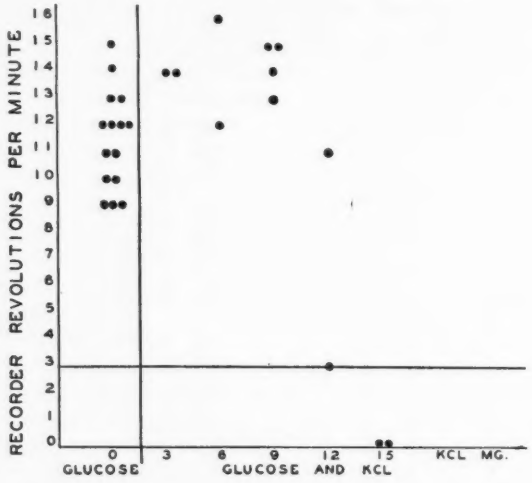

Fig. 5. Effect of 3 cc. of 15% glucose and KCl on the maximum rate of work following infusion. (The 2 animals which received 15 mg. of KCl developed an immediate respiratory and cardiac paralysis and succumbed. This amount of KCl is lethal to some normal rats.)

revolutions per minute respectively. This small difference in averages may have been owing to chance. The data are not presented graphically.

Experiment 6. Thirty adrenalectomized rats were treated with epinephrine in the amount of 0.4 cc. of 1:5000 adrenalin chloride[2] given subcutaneously. It has been demonstrated (9) that this amount was within the range of dosage which gave an optimal increase in the rate of work of adrenalectomized animals under similar conditions. Ten animals received, in addition to the epinephrine, an intravenous infusion of 3 cc. of 2.55% sodium chloride solution, 10 received an intravenous infusion of 15% glucose and 10 animals did not receive any additional treatment.

The results (fig. 4, table 1) confirmed the observations (9) that the subcutaneous administration of adrenalin chloride to the adrenalectomized rat under these experimental conditions improved the rate of work. When an intravenous infusion of 3.0 cc. of a 2.5% sodium chloride solution was given in addition to the adrenalin chloride treatment the average increase in the rate of work was but slightly higher and may have been owing to chance. In contrast, infusions with 3 cc. of 15% glucose solution in addition to the adrenalin chloride treatment gave a significantly greater restoration

[2] Parke, Davis & Co., Detroit, Mich.

in the rate of work so that the average maximum rate of work attained was approximately equal to the rates of work shown by normal rats (fig. 1) at comparable periods of time after the beginning of stimulation.

Experiment 7. The purpose was to determine whether the administration of the potassium ion in addition to glucose would have the effect of inhibiting the beneficial effect of the glucose. Accordingly, 12 adrenalectomized rats were given intravenous infusions of 3 cc. of 15% glucose solution. In 2 animals the glucose solution contained 3 mg. of potassium chloride, in 2 animals it contained 6 mg., in 4 animals it contained 9 mg., in 2 animals it contained 12 mg. and in 2 animals it contained 15 mg.

The results (fig. 5) demonstrated that the beneficial effect on work of infusions with 3 cc. of 15% glucose is not inhibited when 3, 6 or 9 mg. of KCl is added with each infusion. When the amount of KCl was increased to 12 mg. one of the 2 animals receiving these amounts failed to show an increase in the rate of work. Each of the 2 animals which received 15 mg. of KCl with the glucose solutions developed an immediate respiratory and cardiac paralysis and succumbed. In the graph (fig. 5) summarizing this data the values for animals receiving infusions of 15% glucose only in *experiments* 3 and 5 are included for comparative purposes.

DISCUSSION

In this study of the reversibility of the fatigue of the adrenalectomized rat it was found that intravenous infusions with glucose solutions gave a good temporary recovery of the ability of the muscle to work. Adrenalectomized animals develop hypoglycemia under many experimental conditions. Ingle, Hales and Haslerud (8) found that hypoglycemia was associated with the fatigue of the adrenalectomized rat under the experimental conditions used in the present study. This was confirmed by Ingle, Nilson and Kendall (10). Studies (3, 11, 12) have shown that those adrenal steroids which have the greatest effect on the ability of the adrenalectomized rat to work under these conditions have also the greatest effect on the carbohydrate content of the blood and tissues of the rat. It seems probable that the inability of the rat to continue work under these conditions is due in part to the failure of its carbohydrate stores and that the beneficial effect of infusions with glucose is due in part to utilization of the glucose administered.

It is important that infusions with solutions of sucrose were found to be as effective as infusions with solutions of sodium chloride for sucrose is not a normal constituent of the body, it is not utilized by the body when injected into the blood stream and its principal effects when so administered are determined by its osmotic activity. When sodium chloride was administered in a small volume (*experiment 4*) it had a very slight effect on work whereas an equal volume of a glucose solution had a much more marked effect. When equal amounts of these substances were administered in the standard volume (3 cc.) the effects on work were increased proportionally. Clearly the volume of the fluid administered is also a factor in reversing the fatigue state.

Although the loss of the sodium and chloride ions from the blood serum has been claimed to be the principal cause of the symptoms of adrenal cortical insufficiency it is apparent that under these conditions the addition of sodium chloride to the blood has no greater effect on work than the administration of an equal volume of an unphysiological crystalloid of the same osmotic activity. Ingle (13) has reported the failure of the high sodium chloride, low potassium diet to maintain the ability of the adrenalectomized rat to work although this treatment was effective in maintaining growth and a state of apparent good health. Ingle, Nilson and Kendall (10) found that the adrenalectomized, nephrectomized rat fatigued rapidly unless treated with cortin although the

values for serum sodium and chloride did not change from normal under these conditions. The cortin treated adrenalectomized rat was capable of working in a nearly normal manner although the serum values for sodium and chloride were depressed to very low values by intraperitoneal injections of water. These workers as well as Hales, Haslerud and Ingle (1), and Ingle (14) commented on the apparent circulatory collapse which was associated with the fatigue of the adrenalectomized rat. In unpublished studies Ingle and Griffith (15) have found that the blood pressure of the adrenalectomized rat is low at the time of fatigue and that the values obtained for blood volume were below normal. It is not possible to make accurate determinations of blood volume under these conditions but the low values obtained at least emphasize the inadequacy of the circulatory mechanism. The reason for the greater effect of hypertonic solutions on work is not clear. It occurred when the concentration of each test substance was increased. Since glucose and sucrose do not ionize to any appreciable extent the influence of the concentration of the test substance on work cannot be due to an ionic effect. It may have been due to the further dilution of the blood stream by water from the tissues due to the raised osmotic activity of the blood. It is probable that the inability of the rat to work under these conditions is due in part to the failure of circulatory mechanisms and that the beneficial effect of infusions is due in part to the improvement of circulation through the muscle and the organs supporting energy exchange.

The total amount of potassium contained in the blood serum of the rats used in these experiments probably does not exceed 1.0 mg. It was found possible to administer as much as five times this amount of potassium intravenously without inhibiting the beneficial effect of the glucose on work. It cannot be assumed that the potassium remained in the blood stream in these proportions for it is known that potassium may diffuse out of the blood stream even in adrenalectomized animals and that the rate of its disappearance is increased in the presence of excess glucose. But the fact that adrenalectomized animals were able to tolerate the addition of these amounts of potassium without inhibiting muscular contraction renders unlikely the hypothesis (16) that the muscular asthenia of adrenal insufficiency is due to an excess of potassium in the body fluids. In a study of the values of serum potassium in fatigued adrenalectomized rats (10) it was found that the level of serum potassium was higher than normal at the time of fatigue but that in the nephrectomized, working rat still higher values for serum potassium might co-exist with ability to work in a normal manner. Miller and Darrow (17) have reached similar conclusions.

During the first few hours of stimulation the adrenal demedullated rat shows a decrease in the rate of work below that of the normal animal. It is probable that this deficiency in work capacity is due to the absence of the adrenal medulla and its hormone, epinephrine, but direct proof is lacking. Ingle (3) has found that work performance of adrenalectomized rats treated with cortin, corticosterone, 11-dehydro-corticosterone, 17-hydroxy-corticosterone, or 17-hydroxy-11-dehydro-corticosterone is approximately the same as that of adrenal demedullated rats during the first few hours of work. It can be temporarily raised to a normal level by the additional administration of adrenalin chloride to either adrenal demedullated rats or to adrenalectomized rats treated with the secretory products of the adrenal cortex. Treatment of the fatigued adrenalectomized rat with infusions of glucose solutions gave a temporary restoration of the rate of work to approximately the same level shown by adrenal demedullated rats and the additional administration of epinephrine gave a temporary restoration of the rate of work to approximately the same level shown by normal rats.

Radwanska (18) was the first to observe that the administration of epinephrine was effective in temporarily improving the work performance of fatigued adrenalec-

tomized animals. Ingle (14) reported that treatment of the adrenalectomized rat with epinephrine was incapable of prolonging the ability to work of adrenalectomized rats although it would induce a brief partial recovery when administered to the fatigued animal. The mechanism whereby epinephrine offsets fatigue in either adrenalectomized or normal animals is unknown. It is known to exert a pressor effect, to increase the rate of blood flow through muscle and to increase the level of blood sugar. These changes may mediate a part of its beneficial effect but they do not explain the similar beneficial effect of epinephrine on the isolated muscle of either normal or adrenalectomized animals.

It was demonstrated that under the conditions of these experiments the fatigue state of the adrenalectomized rat was reversible. It cannot be assumed that the treatment found effective here would necessarily reverse the fatigue states associated with other conditions of adrenal insufficiency. Since the animals had been deprived of their adrenal glands for a period of only a few hours and since the conditions imposed on the animal are atypical, these experimental animals had little in common with animals and patients having chronic adrenal cortical insufficiency.

SUMMARY AND CONCLUSIONS

A study was made of the reversability of the fatigue of the adrenalectomized rat under certain conditions. The rats were made to work immediately following adrenalectomy by stimulating the gastrocnemius muscle to lift a 100 gm. weight 3 times per second until muscular responsiveness was almost lost. The animal was then treated with intravenous infusions of solutions of glucose, sucrose, sodium chloride, potassium chloride and with subcutaneous injections of adrenalin chloride. Normal rats and adrenal-demedullated rats were made to work for a 10-hour period to establish standards for the work performance of rats having an intact adrenal cortex with and without the adrenal medulla. The following conclusions have been reached.

The work output of adrenal demedullated rats decreased more rapidly than for normal rats during the first 10 hours of stimulation.

In adrenalectomized rats the intravenous administration of glucose in any concentration gave a significantly greater increase in the rate of work than did isomolar solutions of sucrose and sodium chloride.

Infusions with solutions of sucrose and of sodium chloride had similar effects on work.

The work performance of animals treated with infusions of 15% and with 20% glucose was improved so that their values for work fell within the range of performance shown by adrenal demedullated rats.

The higher concentrations of glucose, sucrose and sodium chloride induced the greatest improvement in work output.

The amount of recovery brought about by infusions was related directly to the volume of the infusion.

The subcutaneous injection of adrenalin chloride improved the rate of work significantly. The additional infusion with a solution of sodium chloride produced little or no further increase in the rate of work whereas the additional infusion with a glucose solution gave a significantly greater restoration so that the average maximum rate of work attained was approximately equal to the rate of work shown by normal rats.

Large amounts of potassium chloride in solution with glucose were administered without inhibiting the beneficial effect of the glucose.

From this and earlier studies it was concluded that (*A*) treatment of adrenalectomized rats with infusions of glucose gives a restoration of work to approximately the

same sub-normal level shown by adrenal demedullated rats and by adrenalectomized rats treated with those adrenal steroids which influence carbohydrate metabolism; (*B*) the administration of adrenalin chloride to adrenal demedullated rats or to adrenalectomized rats treated with either adrenal steroid or glucose increases the rate of work to approximately the level shown by normal rats.

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ACTION OF NOCUOUS AGENTS ON INSULIN AND ADRENALIN SENSITIVITY, AND ON GLUCOSE TOLERANCE[1]

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WHEN an organism is continuously exposed to certain damaging agents, such as cold, exercise, various drugs, rays, etc., it usually responds with a syndrome which has been described by Selye (1, 2, 3) under the name of the 'general adaptation syndrome.' The symptoms and signs of this syndrome are largely independent of the specific nature of the agent which elicited them and have consequently been regarded as expressions of the response of the body to damage as such. The syndrome develops in three stages: first, the stage of the 'alarm reaction,' which evolves during the first 24 to 48 hours of exposure, second, the 'stage of resistance,' which attains its maximum after a few days or weeks, depending on the nature and the dose of the eliciting nocuous agent, and third, the 'stage of exhaustion,' at the end of which the animal dies, having apparently exhausted all its adaptability or 'adaptation energy.' The morphological, physiological and chemical changes characteristic of each of the above-mentioned three stages of the general adaptation syndrome have been described elsewhere; suffice it to re-emphasize here that the changes in carbohydrate metabolism are particularly marked, inasmuch as during the alarm reaction pronounced hypoglycemia, preceded by transitory hyperglycemia, is characteristic, while during the stage of resistance the blood sugar rises above normal and is maintained at a high level until eventually, during the stage of exhaustion, fatal hypoglycemia develops (4, 5).

In view of these important blood sugar changes, it appeared of interest to determine the manner in which the general adaptation syndrome would influence the sensitivity to insulin and adrenalin and the glucose tolerance curve. This was deemed particularly important in view of the fact that the response of the blood sugar to damaging agents is entirely non-specific. This means that in the numerous experiments in which the influence of certain drugs on the blood sugar has been studied, a correction must be made for whatever part of that action might merely have been due to the fact that in the doses given they elicited the general adaptation syndrome. These same considerations naturally apply also with regard to agents purported to exert a specific influence on adrenalin or insulin sensitivity and the glucose tolerance curve.

METHODS

For all our experiments we used adult male rats weighing 200 to 250 gm. Since it is known that the diet during the period preceding treatment with insulin, adrenalin and glucose has a considerable effect on the blood sugar response to these agents (6, 7), the animals were kept on a uniform Purina diet, and fasted 24 hours before blood sugar determinations. Sugar was determined by means of the Hartman, Shaffer and Somogyi method in tail blood.

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In order to elicit the general adaptation syndrome, 3 different agents were used, namely, subcutaneous injections of formaldehyde (0.3 cc. of a 10% solution, given 3 times daily), forced muscular exercise (1 hour 3 times daily in a drum cage having a diameter of 12″ and turning at a speed of 16 revolutions per minute) and exposure to cold (animals kept continuously at −2° to +2° C.).

EXPERIMENTAL

Action of insulin. Twenty-four hooded rats were divided into 4 groups of 6. One group remained untreated, and served as a control, the second was treated with formaldehyde, the third forced to perform muscular excercise, and the fourth exposed to cold, in the manner described above. The same animals were used during the alarm reaction, that is 24 hours after initiation of treatment, and during the stage of resistance, on the 6th and 9th day. In each case, the animals were fasted 24 hours, then received subcutaneous injections of 1 U of insulin. Tail blood was taken 30 minutes later for blood sugar determination. The results are summarized in table 1.

TABLE 1. GLYCEMIC RESPONSE AFTER INJECTION OF ONE UNIT OF INSULIN
(Each number represents the average value for 6 rats)

Treatment		Blood sugar			
		Controls	Cold	Exercise	Formaldehyde
		mg.%	*mg. %*	*mg. %*	*mg. %*
24 Hours	(*a*) Initial	78	67	59	78
	(*b*) ½ hr. after insulin	57	48	39	59
	(*c*) Difference	21	19	20	19
6 Days	(*a*) Initial	73	83	75	91
	(*b*) ½ hr. after insulin	56	41	47	61
	(*c*) Difference	17	42	28	30
9 Days	(*a*) Initial	80	100	85	82
	(*b*) ½ hr. after insulin	57	42	55	51
	(*c*) Difference	23	58	30	31

It will be seen that the initial average blood sugar values in the untreated controls vary only between 73 and 80 mg.%, and that in the animals exposed to the various damaging agents the blood sugar values tend to be low after 24 hours (during the alarm reaction) and high on the 6th and especially so on the 9th day (during the stage of resistance). These changes in blood sugar are typical of the first two stages of the general adaptation syndrome, and will be encountered again in the experiments to be mentioned later in this communication. It should be emphasized, however, that the various stages of the reaction do not evolve exactly at the same time in all individuals, and consequently neither the hypoglycemia of the alarm reaction nor the hyperglycemia of the stage of resistance is as clear-cut in these experiments as it is if continuous individual blood sugar curves are considered. Table 1 indicates, however, that even in those cases in which the initial blood sugar does not deviate significantly from the normal, for instance in the formaldehyde treated group, the response to insulin is very different during the stage of adaptation from that during the alarm reaction or in untreated control animals. Thus one may say that insulin resistance is actually a more sensitive index of the stage of adaptation than the fasting blood sugar itself. During the alarm reaction, the insulin hypoglycemia is approximately the same in this experimental series as in not pretreated controls. On the other hand, both on the 6th and on the 9th day of treatment with any one of the three nocuous agents used, the same dose of insulin consistently produces a particularly severe drop in blood sugar. It ap-

pears that the hypoglycemic effect of the hormone increases as the animal adapts itself to these non-specific damaging agents.

Since the determination of a single point of the blood sugar curve cannot give a true picture of the extent of insulin hypoglycemia, we repeated the above experiment in animals in which the blood sugar was determined ½, 2, 4 and 6 hours after insulin administration. Our stock of hooded rats being exhausted at this time, albino rats were used. As these are particularly sensitive to insulin, one-fourth of a unit sufficed to produce significant hypoglycemia after 24 hours of fasting. Since repeated small hemorrhages, such as are unavoidable if blood is taken from the tail, may affect the blood sugar changes, we discarded the animals at the end of each 6-hour curve, hence different groups had to be used for the studies performed during the alarm reaction (24 hours) and during the stage of resistance (9 days).

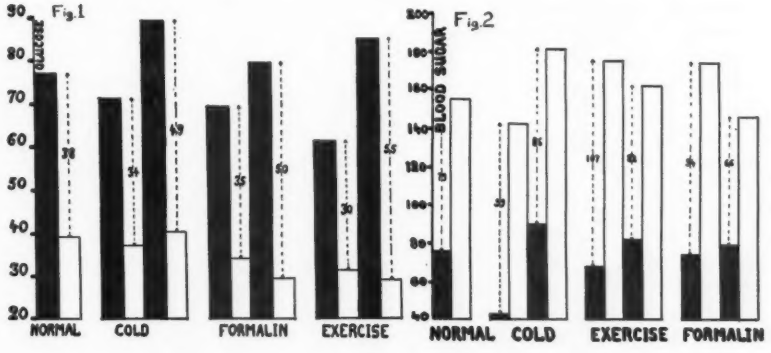

Fig. 1. Action of insulin. Fig. 2. Action of adrenalin. (Black bars—initial glycemia; white bars —maximum response to hormones or glucose. The first two bars of the experimental groups indicate the results obtained during the alarm reaction, and the second two bars the results during the period of resistance. The figures in interrupted lines show the difference between the initial glycemia and the maximum response.)

The slope of the hypoglycemic curve had approximately the same shape in all groups including the controls—the blood sugar reaching its lowest value after about thirty minutes in most cases—hence for the sake of brevity, in figure 1 only the initial blood sugar and the most pronounced hypoglycemic values are recorded.

We note that the initial blood sugar (black columns) is below normal during the alarm reaction and above normal during the resistant stage in all cases, although the deviations are not always very pronounced due to the fact that here again only average values of single determinations are given instead of continuous individual blood sugar curves. As in the first experimental series, the fall in blood sugar caused by insulin is approximately the same during the alarm reaction and in the non-treated controls. The fact that, at least in some groups, it appears slightly subnormal may be due to the somewhat lower initial blood sugar level during this stage. On the other hand during the stage of resistance, the hypoglycemia produced by the same dose of insulin is much more profound than in control animals. This is particularly noteworthy in the formaldehyde treated and exercised groups, in which even the absolute blood sugar values after insulin are lower than in the controls, although the initial glycemia was above normal.

Action of adrenalin. In this experiment we used 2 series each consisting of 4 groups of 6 animals which received the same pretreatment as those described above. One of

these series was then treated with adrenalin (0.2 cc. of a 1/1000 solution subcutaneously, after a 24-hour fast) during the alarm reaction, that is, after 24 hours' pretreatment with the nocuous agents, and the other series, after 9 days of pretreatment, during the stage of resistance. The results are summarized in figure 2.

The relatively low initial blood sugar values during the alarm reaction and the relatively high glycemia of the resistant stage are again in evidence. It is also quite obvious from the graph that during the alarm reaction adrenalin causes a much more pronounced rise in blood sugar than in the non-treated controls. During the resistant stage, on the other hand, the changes are more difficult to evaluate. If only the maximum hyperglycemia is compared in each group (as has been done in the graph) no significant change is noted. We may say, however, that a comparison of the whole blood sugar curves—which are not recorded here—shows certain differences; in the

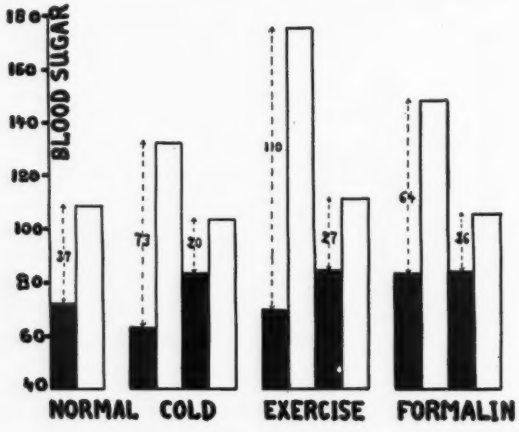

Fig. 3. Glucose tolerance

controls during the alarm reaction there is a transitory decrease in the adrenalin hyperglycemia after 5 hours, which is followed by a second fairly prolonged rise. On the other hand, during the stage of resistance, once the peak of the hyperglycemia is reached, the blood sugar decreases continuously until it reaches the normal level.

The glucose tolerance curve. Here again we used 2 series each consisting of 4 groups of 6 animals, which received the same pretreatment as those previously described. The glucose tolerance of these series was tested after 24 hours and after 9 days of pretreatment respectively, 1 cc. of a 50% glucose solution was administered by stomach tube for tolerance tests after a 24-hour fasting period.

A comparison of the average initial blood sugar values with the average maximum hyperglycemia as recorded in figure 3 clearly indicates that during the alarm reaction glucose tolerance is decreased while during the period of resistance it is increased.

DISCUSSION

Numerous authors have described changes in the glycemic response to insulin caused by agents which in the light of our present-day knowledge obviously must have elicited a general adaptation syndrome.

Lawrence and Buckley (8) reported that after injection of diphtheria toxin the action of insulin is reduced or absent. Similar findings were reported in experi-

mental anemia in the rabbit (9), in artificial fever (10, 11), and after muscular exercise (12). In opposition Sendrail and Blancardi (13) found in the dog that there is a proportional ratio between the length of exercise and the fall of the blood sugar after injection of insulin.

In regard to adrenalin, Sakai (14) reported its action in trained and untrained dogs. In both cases, there was an increased sensitivity to adrenalin, which was more marked in the untrained animals than in the trained.

The glucose tolerance test has been extensively used after the action of different agents: in chloroform and phosphorus poisoning (15), in diphtheria toxemia (16, 17, 18), after exercise (14), in fever (11), and in infectious diseases (19). In acute intoxication with phosphorus and chloroform there is a diminution of the glucose tolerance, while during recovery the tolerance returns to normal. After progressive intoxication with diphtheria toxin, Soskin and Mirsky reported that the sugar tolerance goes through the following stages: normal, diabetic type, normal and abnormal. Sakai studied the glucose tolerance after exercise in trained and untrained dogs: in untrained, the tolerance is greatly diminished, while in trained, the tolerance is too inconstant to evaluate. During infectious diseases, Labbé and Boulin reported a decrease in glucose tolerance which persists for a certain time after cure.

All our results are in agreement with these observations, especially with regard to the changes during acute damage which correspond to the alarm reaction. The slight differences between our observations and those of others concerning insulin hypoglycemia are probably not very important. For example Lawrence and Buckley found a considerable increase in insulin resistance during diphtheria toxemia while we noted only a very slight decrease in the hypoglycemic action of this hormone during the alarm reaction. This difference may, however, merely be due to the fact that the above-mentioned investigators used doses so high that all their animals died within 24 hours. The same explanation may also apply to the observations concerning very acute phosphorus and chloroform intoxication. It is noteworthy, however, that even an alarm reaction insufficient to raise considerably the insulin resistance causes a very pronounced rise in the hyperglycemic action of adrenalin and of glucose. This observation appears to indicate—contrary to views previously expressed (20)—that animals resistant to adrenalin are not always much more sensitive to insulin.

SUMMARY

In rats continuously treated with various damaging agents (exposure to cold, forced muscular exercise, formaldehyde injections), there develops an initial period of hypoglycemia (alarm reaction) followed by a second period of hyperglycemia after a certain degree of adaptation is acquired (stage of resistance). This confirms previous work along these lines indicating that during adaptation to damaging agents certain biochemical changes develop which appear to be entirely independent of the specific nature of the damage and are apparently corollaries of adaptation as such.

During the alarm reaction, after 24 hours of treatment with the above-mentioned damaging agents, a small and perhaps insignificant decrease in the hypoglycemic action of insulin was noted. This was accompanied by a marked increase in the hyperglycemic action of adrenalin and orally administered glucose.

During the stage of resistance, after 9 days of treatment, the hypoglycemic action of insulin and the alimentary hyperglycemic response were considerably increased, while the adrenalin sensitivity showed rather irregular changes.

Comparison of these findings with the existing data concerning the action of acute and chronic damage on the glycemic response to insulin, adrenalin and glucose, shows that changes in the response to these agents cannot be regarded as specific pharmaco-

logical actions without considering the possibility that they may merely be the result of adaptation to the non-specific damaging effect of such agents.

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SOME EFFECTS OF CONSTANT INTRAVENOUS INJECTION OF POTASSIUM CHLORIDE SOLUTIONS INTO NORMAL CONSCIOUS DOGS

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THE MAJORITY of workers are in disagreement as to the nature of the fundamental cause or causes of adrenal insufficiency. The most widely held theory is that the syndrome is due to some defect in salt and water metabolism. Some have suggested that defective sodium metabolism is the primary cause (1, 2, 3); others, that it is a failure of the blood-diluting mechanism (4, 5, 6, but see also 7); and still others, that the syndrome is due to hyperpotassemia (8, 9, 10).

Various workers have induced some of the symptoms of adrenal insufficiency by KCl administration, but the majority of the authors have not examined their animals or experimental subjects on more than a few of the facets of the problem. Zwemer and Truszkowski (11) present the most comprehensive report to date, but their report lacks details of experimental technic and treats only subjectively of the symptoms of adrenal insufficiency. In their experiments, after intraperitoneal injections of KCl solutions, the blood potassium level of otherwise normal cats rose and was maintained at high levels by subsequent injections. Anorexia was present when the serum potassium was 30 mg.%, severe muscular asthenia appeared when the serum potassium was above 40 mg.%, convulsions were obtained at about 50 mg.%, and death occurred at 60 mg.%. In measuring the rate of disappearance of potassium from the blood stream, Whelan (12) injected 10% KCl intravenously into dogs and found an increase in urine sodium like that of adrenal insufficiency. In normal human beings, intravenous injections of KCl solutions produced hemoconcentration, a symptom prominent in Addison's disease (13). Winkler et al. (14) injected isosmotic KCl solutions (intravenously) into normal dogs and obtained electrocardiographic records of some of the phenomena reported in dogs with adrenal insufficiency (15). In acute experiments, Hastings and Compere (8) injected KCl intravenously into dogs and reported that death occurred when the potassium in the serum reached 80 mg.%, a concentration lethal to the mammalian heart in vitro (16) and in vivo (14). Low blood sugar values after injection of potassium salts have been observed (17). Keith and Binger (18) reported the experimental induction of high levels of serum potassium in normal human beings and the presence of equally high potassium levels in diseased conditions without concomitant clinical symptoms of potassium poisoning. Thus it can be seen that each single report is concerned with only a few of the changes due to hyperpotassemia. In the experiments to be described, therefore, an attempt has been made to record quantitative as well as subjective data on as many different aspects of adrenal insufficiency as possible.

It should be noted that most of the reports in the literature are concerned with acute experiments in which the injections of potassium were made during a few minutes or a few hours. In our preliminary experiments an attempt was made to maintain constant intravenous injections of KCl solutions in such amount and concentrations that the individual dogs would live as long as untreated adrenalectomized dogs. It was found that 6 to 8% KCl injected at a rate of 6 cc./hr. was lethal after 2 to 12 days.

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The injection of 6 cc./hr. produced a minimum of blood dilution. Despite the fact that the injected material has an osmotic strength 4 to 6 times as great as that of blood, no hemoglobinuria was observed at any time.

METHODS

A dog was lightly anesthetized with pentobarbital sodium (Nembutal), 32 mg./kg., and a small longitudinal incision was made in the neck near the jugular vein, rigid asepsis being observed. A white rubber tube of standard make (3/16 in. diameter with 1/16 in. bore)[1] was put into the jugular vein proximal to a tight ligature and was pushed cardiad for 6 or 8 inches. This tube, serving as an elongate cannula, was tied in place by two tight ligatures around the vein wall, leaving 5 or 6 inches of tube outside the vein. A piece of ordinary tire-patching was cemented on the tube about 3 inches from the external end. A needle was threaded, using a foot or so of linen thread which was tied to the end of the tube. This needle was pushed through the subcutaneous spaces and out through the skin about an inch caudad to the inion. Thus, when the thread was pulled, the tube followed it to a subcutaneous position near the middle of the back of the neck. The incision near the jugular vein was closed, and the dog was bandaged and allowed to recover.

On the third postoperative day, the bandage and stitches were removed. After local anesthesia had been induced in the skin at the back of the neck, the thread was pulled until the tube was brought out of the dog's body to the point at which the tire-patching was directly beneath the skin. In this position, the tire-patching prevented the tube from pulling out of the animal. Another piece of tire-patching was cemented to the tube outside of the skin to prevent the tube from slipping back beneath the surface. Through this tube, intravascular injections of any amount of fluid could be made at any desired rate. Blood samples could be withdrawn through the tube rapidly and efficiently. When a sample was to be drawn, about 1 cc. of Ringer's solution was injected through the tube and then 1 cc. or more of blood slowly withdrawn before the sample was collected. The whole process of injection and sample collection was painless, simple and convenient.

The tube was held in one position on the dog's neck by a heavy leather collar. By means of a constant injection pump, fluids were injected into a system of rubber tubes which led into the intravenous tube. The rate of injection could be varied from 1 to 40 cc. per hour. In all the experiments here described, a rate of about 6 cc./hr. was used, thus preventing undue blood dilution. The dogs were kept in metabolism cages. A chain from the side of the cage to the dog's collar allowed free but not excessive movement.

Determinations were made of the serum and urine content: (*a*) of potassium, by the microphotoelectric method of Hoffman (19); (*b*) of sodium, by the method of Hoffman and Osgood (20); (*c*) of chloride, by the method of Patterson (21); (*d*) of urea, by the method of Van Slyke (22) and (*e*) of glucose, by the method of Miller and Van Slyke (23). Rectal temperatures were recorded in some instances, and hematocrit determinations were made by centrifuging citrated or heparinized blood in capillary tubes.

RESULTS

The data on 6 dogs are presented in tables 1 through 6. The urine volumes reported below are those collected as the dog voided them. Each sample was analyzed as a unit.

[1] Manufactured by Salisbury Rubber Company.

TABLE 1. PROTOCOL ON *dog 1*, INJECTED WITH 6% KCl AT A RATE OF 6 CC./HOUR

Hr. Inj.	Hemato-crit	Serum			Urine							
		K^+	Na^+	Cl^-	Vol.	Per hr.	K^+	K^+	Na^+	Na^+	Cl^-	Cl^-
	% cells	mg.%	m.eq./l.	m.eq./l.	cc.	cc.	mg.%	gm.	m.eq./l.	m.eq.	m.eq./l.	m.eq.
0	38.8	17.5	156	102.6								
4½	43.5	13.8	171	85.9								
17½	42.7	19.0	168	103.7								
20½		20.5										
42½	26.1	19.0	165	102.8	250	4.7	2580	6.45	64	16.0	233	58.3
52½	27.8	17.8	168	99.1								
72					200	7.0	3640	7.28	34	6.8	166	33.2
78½	29.9	28.7	168	98.8								
90½		23.8	166	96.4	215	11.3	3450	5.27	12	2.5	406	87.3
101½	29.9		170	102.3								
111½	Found Dead				188	17.9	2260	4.25	11	2.0	526	98.9

The last serum sample from *dog 2* was drawn at the very moment of death. The injection pump had been disconnected 5 to 7 minutes before the heart ceased to beat, so blood from the auricle (for autopsy showed this to be the location of the end of the tube) was a well mixed sample.

None of the gross symptoms of adrenal insufficiency was seen. At no time did the animals exhibit the asthenia reported in cats (11) and in dogs (8). It should be emphasized that the dogs were visited at least once each 5 hours so that there could have been no severe or prolonged collapse or asthenia without its having been noticed and recorded. The interesting dog in this connection was *dog 2*. This dog remained in good spirits until after urine *sample 8* was collected. A severe lassitude, but no weakness, appeared during the following 2 hours. The tube was disconnected from the injection pump and several minutes passed before the last blood sample was drawn. And in the next 5 minutes the dog collapsed and died. The reactions of the dogs to the manipulations necessary to obtain rectal temperatures were normal. Although they did become accustomed to the handling, muscular tension was always present and the dogs promptly jumped to their feet when the thermometer was withdrawn. There was no sign of the convulsions described by Zwemer and Truszkowski (11).

The dogs were offered a large bowl of cooked beef lung and bread at the same time each day, and in no case, except within a few hours of death did the food go untouched. The daily food consumption toward the end of the experiment was the same as during the control period, and the daily ration was more than enough to maintain body weight. In contrast to the findings of Zwemer and Truszkowski (11) in cats, these dogs had no indication of anorexia. Although no quantitative tests were

TABLE 2. PROTOCOL ON *dog 2*, INJECTED WITH 8% KCl AT A RATE OF 6 CC./HOUR

Hr. Inj.	Serum			Urine				
	K^+	Na^+	Urea N	Vol.	Per hr.	Na^+	Na^+	Urea N
	mg. %	m. eq./l.	mg. %	cc.	cc.	m. eq./l.	m. eq.	mg.%
0	17.5	161	19.5	295	6.1	77	22.7	749
2				55	27.5	40	2.2	874
6	31.2	154	14.6	85	21.2	177	15.0	297
12½	24.1	148	12.2	100	14.3	72	7.2	228
18½	30.6	175		45	7.5	43	1.9	689
33½				143	9.5	65	9.3	802
40½	32.6	156		100	14.3	73	7.3	925
43½				78	26.0	80	6.2	955
45	58.1	150	27.5	45	22.5	90	4.1	817
46	Died							

TABLE 3. PROTOCOL ON *dog* 3, INJECTED WITH 6% KCl AT A RATE OF 6 CC./HOUR

Hr. Inj.	Serum		Urine					
	K^+	Na^+	Vol.	Per hr.	K^+	K^+	Na^+	Na^+
	mg.%	*m.eq./l.*	*cc.*	*cc.*	*mg.%*	*gm.*	*m.eq./l.*	*m.eq.*
0	17.8	166	185	7.7	250	0.46	13	2.4
8	15.5	153	55	9.2	760	0.7	94	8.7
21¼	19.0	150	300	23.0	440	1.3	141	42.3
33¼	27.5	152						
81	20.1	160	380	6.3	1700	6.46	93	35.3
105	25.3	186						
119½	24.0	176	230	6.0	1560	3.58	75	17.3
142¼	20.0	148	280	11.3	3800	10.64	59	15.3
154¼	30.0	140	185	15.4	1850	3.32	29	5.4
165	21.2	132	46	4.2	3110	1.43	19	0.9
175	34.8	134	217	21.7	4040	8.77	46	10.0
190¼	50.5	142	243	16.2	2090	5.08	67	16.3
217¼	42.0	148	690	25.5	1090	7.49	65	44.8
238			375	17.9	2750	10.3	25	9.4
266	39.3	152	375	13.4	3725	13.97	33	12.4
269¼	Found Dead							

performed, as far as one could see, the dogs were perfectly normal with regard to appetite, posture, and general behavior.

DISCUSSION

The theory that the symptoms of adrenal insufficiency are due to potassium poisoning is not upheld by the data. Objectively, the only symptom indicated by the

TABLE 4. PROTOCOL ON *dog* 4, INJECTED WITH 6% KCl AT A RATE OF 6 CC./HOUR

Hr. Inj.	Rectal temp.	Serum				Urine					
		K^+	Na^+	Cl^+	Urea N	Vol.	Per hr.	K^+	Na^+	Cl^-	Urea N
	°C.	*mg.%*	*m.eq./l.*	*m.eq./l.*	*mg.%*	*cc.*	*cc.*	*mg.%*	*meq./l.*	*meq./l.*	*mg.%*
◻		9.2	162	91.0	18.8						
0	40.0					235	14.2	460	22	42.6	124
12	40.5	23.0	174	91.5	27.5	230	11.3	1930	154	80	865
16½	40.3					230	51.1	3480	140	244	820
20½	39.8										
24	40.2	20.8	161	93.0	26.9						
33¾	40.0					280	16.4	4600	60	396	859
37½	40.0	33.6	170	93.1	22.1	83	20.8	2625	61	384	598
48	40.0	22.5	153	95.4	24.0	70	6.6	5400	57	378	688
57½						66	6.9	4745	82	416	1107
60		20.0									
65½	39.0					120	15.0	2315	91	455	765
71½	39.3	21.1	156	104.0	28.8	165	26.0	2170	84	426	434
81½						110	11.0	6080	89	369	1039
84		24.9	169	109.0	23.4						
94½	39.6					150	11.5	2800	43	345	623
96	39.0	11.2	156	103.0	27.7						
106½	39.5					130	10.8	3380	43	165	763
108½	39.6	28.2	190	123.0	23.7	110	55.0	4800	70	400	651
117						70	8.2	4200	40	275	778
120	40.0	16.3	154	107.0	29.2						
131½	39.5	34.3	174	121.0	21.2						
135						205	11.3	5220	71	330	1400
144		28.0	180	115.0	21.0	125	14.0	3600	100	410	739
156	Found Dead					110	9.2	5350	191	390	1300

data is a high urine sodium. The serum sodium was not consistently altered. The sodium level in the serum of *dog 1* was above the normal level during most of the period of injection, that of *dog 3* was below the normal level; and those of *dog 2, 4, 5* and *6* varied, being sometimes above and sometimes below the normal value. These data do not confirm the contention of Blum et al. (24) that serum sodium falls after the administration of potassium, but are in accord with the work of Whelan (12) who found that intravenous injections of KCl reduced the serum sodium of only one dog in six. The high urine sodium following administration of potassium salts confirms the work of many authors (12, 25). The data show that although there were rises in urine sodium concentrations and in sodium excretion rates (milli-equivalents per hour) dur-

TABLE 5. PROTOCOL ON *dog 5*, INJECTED WITH 6% KCl AT A RATE OF 6 CC./HOUR

Hr. Inj.	Rectal temp.	Hemato-crit	Serum					Urine						Ate food
			K^+	Na^+	Cl^-	Urea N	Glu-cose	Vol.	Per hr.	K^+	Na^+	Cl^-	Urea N	
	°C.	% cells	mg.%	m.eq./l.	m.eq./l.	mg.%	mg.%	cc.	cc.	mg.%	m.eq./l.	m.eq./l.	mg.%	
0	39.0							160	11.0	100	101	91	1318	
0	39.3							230	21.0	35	83	122	934	
0		37.6	15.0	135	103.1		84							*
0	39.2	39.8	14.3	140	94.9	17.4	82							
0	40.0	38.7	14.6	134	102.7	23.8	71	260	20.8	40	65	116	470	
0	39.0	38.9	15.0	140	97.3	22.3	92	250	17.9	17	62	93	847	*
0	39.1	35.9	14.3	138		10.2	91							
0	38.9	40.5	10.7	136	101.4	27.8	101							
12½	40.9	37.9	19.3	139	77.9		83	185	8.2	980	122	399	1130	*
20½	40.9							80	10.0	850	113	364	305	
22½	40.9							40	20.0	400	37	208	197	
24½	41.0	35.3	14.4	129	91.6	13.5	97							
35½	40.1	33.2	17.0	135	94.7	11.1	69	125	9.7	1035	56	940	573	*
43	40.9							115	15.2	1375	15	1035	372	
48		34.5	15.9	129	93.1	11.9	85							
54½	40.5							105	9.0	1520	17	795	1048	
60½		25.5	16.9	131	93.0	8.2	91							
62½	40.1							225	39.0	51	2	437	23	*
70½	41.0							110	13.6	1405	42	119	390	
72½		32.3	25.8	143	111.7	12.6	82							
81½		31.8	15.2	143		12.6	109							
86½								120	7.5	1500	35	595	1290	
92½	40.0	35.2	19.7	141	100.6	15.6	55							
98	40.4							157	12.6	1335	34	610	1185	
101½	40.9	33.5	18.9	132	90.7	11.8	45							
103½								100	18.2	1250	32	800	709	
111½		38.0	33.1	119	93.3	22.5	86							
114	37.9							50	4.8	1700	9	405	760	
117								38	12.2	1480	8	292	940	
121½	36.4	30.6	39.0	145	98.2	32.4	137							
127½								110	10.0	1550	12	367	725	
131	37.0	26.7	38.8	147	104.5	40.1	143							
135½			Died					40	4.5	355	17	182	245	

ing the early hours of injection, both these values later decreased to the normal range or below. The animals continued to eat their daily rations of cooked beef lung and bread, so that the incoming sodium was normal in amount. Thus, although there was a tendency toward a negative sodium balance like that observed in adrenal insufficiency, the animal itself corrected the condition before any of the symptoms of sodium deficiency appeared. The dogs did not show the asthenia described in human sodium depletion (3).

The serum sodium values ran somewhat higher than those reported by most workers (26, 27), although they are in the range reported by Whelan (12). We are at a loss to account for these high values as: the same range of values were obtained in completely normal dogs, so evidently the technic itself was not responsible; nor could any sodium be found in the mineral oil under which the blood was collected; nor does it appear to be the chemical method, for rat serum, using this method, gave values of about 145 milli-equivalents per liter which level is normal for this species. It is clear, at any rate, that no consistent drop in the sodium concentration in the

serum, as we were measuring it, occurred. It was observed that there was no consistent relationship between serum sodium and potassium changes.

It can be seen that it was very difficult to increase the serum potassium by a very great margin. Even the injection of KCl in such amounts as were lethal in 48 hours failed to maintain the serum potassium much above 30 mg. % until near death.

In 1883, Bochefontaine (28) found that the injection of 2 gm. of potassium salts would kill a dog. A more recent study (14) showed that the injection of 10 cc. of 1.12% KCl solution per minute would kill their dogs in about 50 minutes. With the constant injection pump employed in our experiments, a much larger amount of potassium had to be injected before the animals succumbed. The data on *dog* 3 shows that about 400 mg. KCl were injected each hour. Since the dog survived 265 hours of injection, this would make the lethal amount of KCl 90 gm. The difference between our results and those of previous workers probably can be explained on the basis of rate of injection. At the moderately slow rate of injection used the animals excreted enough

TABLE 6. PROTOCOL ON *dog* 6, INJECTED WITH 6% KCl AT A RATE OF 6 CC./HOUR

Hr. Inj.	Rectal temp.	Hemato-crit	Serum					Urine						Ate food
			K^+	Na^+	Cl^-	Urea N	Glu-cose	Vol.	Per hr.	K^+	Na^+	Cl^-	Urea N	
	°C.	% cells	mg.%	m.eq./l.	m.eq./l.	mg.%	mg.%	cc.	cc.	mg.%	m.eq./l.	m.eq./l.	mg.%	
0		38.8	13.6	162	95.3	25.9								
0		36.8	13.8	153	85.6	26.0								•
0	39.6	34.6	10.8	164	81.8	26.2	96							
0	40.1	33.4	12.2	163	91.8	26.2	79	135	9.0	16	3	18.7	1600	
11	40.3							110	24.4	117	3	4.0	2354	•
0	40.1	31.3	11.0	162	92.7	26.4	91	135	19.4	36	1	19.5	1468	
12	40.0	33.1	15.1	162	94.1	13.9	67							
15½								85	5.5	480	8	180.8	1440	
21½	40.9							200	33.3	635	9	102.2	296	
23½		33.7	17.3	163	91.7	12.3	72							
34	40.2							150	12.5	920	9	304.7	1419	
36½		34.2	15.5	145	91.1	18.8	69							•
48	40.2	37.2	19.0	137	91.1	19.3	85							
54		Died						170	8.5	805	6	360.5	1236	

potassium to survive much longer periods. Undoubtedly much smaller amounts would have killed the dogs had the injections been made suddenly. As an example of the importance of the rate of injection, Winkler et al. (14) report a dog which survived 4.29 gm. of KCl (in isosmotic solution) injected in 54 minutes, but which succumbed at a later date to a much smaller amount, 0.89 gm. (as 9% KCl) when the injection was made in 3 minutes.

The hematocrit values as reported in the tables obviously indicate no hemoconcentration, on the contrary, a slight dilution is evident. This result fails to confirm the work of Levy (13) on normal human beings after potassium injections.

The values for serum glucose show fairly large, but not unusual variations. As can be seen from the tables, the serum glucose rose following the consumption of meals all of which contained bread. Most significant is the fact that at no time did the blood sugar reach dangerous or convulsive levels (25–35 mg.%).

There was no great impairment of the ability of the kidneys to concentrate urea, nor did the excretory rate vary significantly from the normal. Despite the great load of potassium thrust upon the kidneys, the serum urea levels remained reasonably constant. This may be taken as definite evidence that the kidneys were not injured by the potassium.

An attempt has been made to correlate urine volume with the other data collected, but no definite relations could be found. From the data on *dog* 2, it appeared as if the urine volume could be correlated with sodium concentration in the urine, but the records of the other dogs showed that no such correlation could be made. Not even by

plotting the observed urine volumes as percentages of the normal could the urine volume, as recorded, be related to the serum or urine content of potassium, sodium, chloride, or urea. The data certainly do not indicate anuria with high serum potassium (29) nor was there a marked diuresis (12, 30). The small increase in urine volume was of the same order of magnitude as that found in man by Keith and Binger (18) after oral administration of KCl.

The analyses for serum and urine chloride led to no definite conclusions. The urinary chloride accounted for less than one-half of the urinary potassium, thus leading to the assumption that much of the base in the urine was balanced by other anions, probably by bicarbonate. *Dog 4*, for instance, excreted about 1600 m.eq. of potassium and only about 750 m.eq. of chloride during the period of observation. These data not only fail to account for the anion accompanying the urinary base, but fail to account for one-quarter of the thousand or so milli-equivalents of chloride that were injected. The 250 m.eq. of chloride injected but not recovered in the urine might have: (*a*) remained in the blood stream, although the data do not indicate this; or (*b*) been excreted by way of the feces, although Harrop (25) thinks this unlikely; or (*c*) been stored in the tissue cells, but Bourdillon (31) and Hastings and Eichelberger (27) have reported data that render this unlikely. More complete analyses might have shown the location of the last one-fourth of the injected chloride.

Our work lends no support to any existing theory of adrenal insufficiency, but it does throw serious doubt on the theory that potassium poisoning is the responsible factor. In confirmation of this, several instances of crisis, shock, or collapse with normal or low potassium levels in the serum, both in experimental animals with adrenal insufficiency and in Addison's disease, were mentioned in the discussions at Cold Spring Harbor. Without directly confirming any theory, our work indicates that the rôle of potassium in adrenal insufficiency is probably a secondary one, perhaps like that suggested by Britton, Silvette and Kline (26). These authors propose a theory that regards the elevated serum potassium as secondary to a change in permeability of tissue cells (as a source of the potassium), concomitant with changed renal function (accounting for the prevention of potassium loss). They ascribe all the symptoms of adrenal insufficiency to the fundamental change in membrane permeability, while our work merely indicates that the symptoms cannot be considered to be the result of the elevated serum potassium.

Now let us consider the results presented in this paper in the light of those reported by Richter and Eckert (32), Rubin and Krick (33), and Sandberg, Perla and Holly (34). Richter and Eckert found that the adrenalectomized rats, given their choice of solutions of several salts of sodium, KCl and distilled water, 'chose' to drink more KCl solution than did normal rats. That these rats chose well is shown by the fact that their choice maintained their lives. It would appear that the salt hunger of adrenal insufficiency extends not only to NaCl and Na lactate, but also to KCl in measurable amounts. Rubin and Krick showed that normal rats, eating only as much as adrenalectomized rats, had negative balances of sodium, potassium and chloride, which facts are the basis of their explanation of the high serum potassium as a secondary phenomenon. Sandberg, Perla, and Holly demonstrated that, of the food they ate, adrenalectomized rats retained less potassium than did normals. In other words, the excretion of exogenous potassium is no problem to the adrenalectomized rat. The 'hunger' of adrenalectomized rats for potassium salts, the explanation of their negative salt balance by comparison with normal rats, and our inability to produce the significant symptoms of adrenal insufficiency in dogs by injection of potassium salt, minimize the importance of the rôle of potassium as a primary factor in adrenal insufficiency.

SUMMARY

A technic for constant intravenous injections into normal conscious dogs is briefly described. Solutions of KCl were injected by this technic into 6 dogs, leading eventually to their deaths in from 2 to 12 days. Determinations of blood concentration (hematocrit), body temperature, and serum and urine content of potassium, sodium, chloride, glucose and urea nitrogen were made at intervals throughout the period of the experiment. The dogs showed only one of the symptoms of adrenal insufficiency, namely, an increase in urine sodium. But this change cannot be regarded as of much significance as it was only temporary. No anorexia, asthenia, or convulsions were observed in any of the animals. From these data, it seems doubtful that potassium poisoning is the primary cause of the symptoms of adrenal insufficiency in the dog.

The author wishes to express deep appreciation and gratitude to Dr. H. G. Swann for his valuable assistance, to Dr. A. J. Carlson for his stimulating suggestions and to Miss Leonore Cohn for technical assistance.

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THE INFLUENCE OF THE THYROID ON THE VAGO-INSULIN AND SYMPATHETICO-ADRENAL SYSTEMS[1]

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SYMPTOMS indicating increased autonomic activity are very common in clinical and experimental thyrotoxicosis. They have been explained largely on the basis of the increased sensitivity of the peripheral autonomic effectors to adrenalin and to the increased metabolism of the various tissues. In view of the fact that the thyroid hormone has a profound influence on the activity of the central nervous system as shown by the study of conditioned and spinal reflexes in normal, thyrotoxic and thyroidectomized animals (1) and by the effect of the thyroid hormone on the respiratory center (3), it was thought of interest to investigate the relation of the thyroid hormone to the autonomic centers regulating the secretion of insulin via the vagus and that of adrenalin through sympathetic nerve fibers.

It was shown previously (4) that various drugs and procedures including metrazol, electrically induced convulsions and typhoid-paratyphoid vaccine, act on both vago-insulin and sympathetico-adrenal centers. This was proven by the study of the effects of these drugs and procedures on the blood sugar in normal, vagotomized, adreno-demedullated, and adreno-demedullated-vagotomized rats. It was found that these procedures elicited a hyperglycemia in the normal and a hypoglycemia in the adreno-demedullated animal. This result was interpreted to mean that both vago-insulin and sympathetico-adrenal systems are excited by metrazol convulsions and typhoid vaccine, and that the effect on the sympathetico-adrenal system supervenes and completely masks the effect on the vago-insulin system. If, however, the adrenals have been demedullated so that adrenalin can no longer be activated by nervous impulses the effect on the vago-insulin system predominates and causes hypoglycemia. The secretion of insulin is under these conditions exclusively controlled by the vagus since metrazol and the other procedures do not have any effect on the blood sugar in adreno-demedullated animals in which the vagi have been cut below the diaphragm. A further proof for the stimulation of the vago-insulin system in the normal animal is seen in the fact that the hyperglycemia produced by these procedures is greater in the vagotomized than in the normal animal because in the former the neurogenic secretion of insulin is no longer possible. The consistency of these results makes it possible to investigate physiological factors which determine the reactivity of the autonomic centers regulating the secretion of insulin and adrenalin respectively. The effect of the thyroid hormone on these centers is the subject matter of this paper.

METHOD

Experiments were performed on rats which were divided into two groups. In the first group the vagi were cut below the diaphragm. In the second group the adrenals

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were demedullated and the animals allowed to drink a 2% NaCl solution. The results obtained with these rats were compared with those obtained in rats in which in addition to the operations mentioned either the thyroid had been removed or thyroxin had been administered for various periods of time. The rats were fasted for 16 hours prior to the experiment. Metrazol was injected subcutaneously; in most experiments 55 mg. per kg. of body weight was administered, in one series only 40 mg. per kg. was given. The electric shock was applied by means of a General Electric Variac of which the primary was connected to the 110 V, 60 CPS line and the secondary was

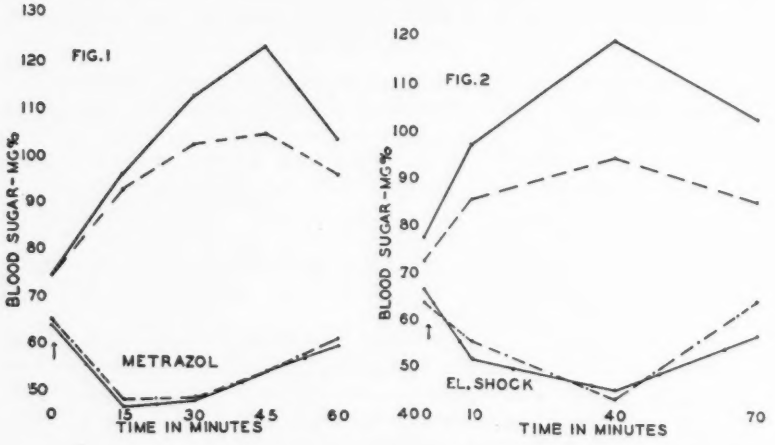

Fig. 1. Effect of metrazol (55 mg./kg. subcut.) on the blood sugar of the rat. (Solid line, vagotomized; dashes, vagotomized-thyroidectomized; dashes and dots, adreno-demedullated; lines with dots between points, lower curve, adreno-demedullated-thyroidectomized). Fig. 2. Effect of electric shock applied to the head on the blood sugar of the rat.

set at 40 volts. The electrodes were applied to the skull and the shock lasted for 0.5 seconds. Typhoid-paratyphoid vaccine[2] was injected intraperitoneally (07 cc./kg.).

RESULTS

Influence of thyroidectomy on the centers of the vago-insulin and sympathetico-adrenal systems. Table 1, A, and figure 1 show the effect of thyroidectomy on vagotomized and on adreno-demedullated rats with respect to their reactivity to metrazol as measured by the blood sugar response. The table shows clearly that the hyperglycemia, particularly 30 and 45 minutes after the injection, is decidedly greater in the vagotomized than in the vagotomized-thyroidectomized rat. The differences are statistically significant. On the other hand, table 1 shows also that the effect of metrazol on the vago-insulin system as revealed by the hypoglycemia produced in adreno-demedullated rats is not influenced by the removal of the thyroid. The means of the blood sugars of the adreno-demedullated and of the adreno-demedullated-thyroidectomized rats are practically identical.

Table 1, B, and figure 2 show the results of similar experiments in which electrical shock was used as a stimulus to test the excitability of the autonomic centers (cf. Kessler and Gellhorn). The results are similar to those found in the metrazol experi-

[2] Supplied by the Abbott Laboratories, North Chicago, Ill.

Table 1. Summary of experimental data.

	Vagotomized rats	Vagotomized rats on thyroxin	Vagotomized thyroidectomized rats	Vagotomized thyroidectomized rats on thyroxin	Adrenodemedullated rats	Adrenodemedullated rats on thyroxin	Adrenodemedullated thyroidectomized	Adrenodemedullated thyroidectomized on thyroxin
	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %
A. Metrazol (55 mg./kg. subcut.)								
No. rats	6		6	6	6		6	4
Control	74.2±2.0		74.1±1.2	75.9±2.1[1]	65.0±1.8		63.8±1.9	67.7±1.7[2]
15 minutes	95.9±3.4		92.6±4.7	96.9±2.0[1]	47.1±2.1		46.6±2.7	48.3±2.1[2]
30 minutes	112.4±3.8		102.2±4.1	115.9±2.7[1]	47.6±2.5		47.1±1.7	44.6±1.2[2]
45 minutes	123.1±5.8		104.7±4.8	124.5±2.5[1]	54.3±2.6		54.4±2.0	52.1±1.9[2]
60 minutes	103.6±4.1		96.0±4.6	112.3±2.6[1]	61.2±1.6		59.6±1.2	59.1±1.3[2]
B. Electric shock (0.5 second)								
No. rats	6		6		6		6	
Control	77.4±1.4		72.3±1.6		63.4±2.1		66.4±1.1	
10 minutes	97.1±1.7		85.6±4.0		55.5±3.5		51.6±3.2	
40 minutes	119.3±2.0		94.6±3.3		43.5±5.6		45.1±1.1	
70 minutes	106.2±4.0		85.3±3.2		64.4±4.0		57.1±2.8	
C. Typhoid-paratyphoid vaccine (0.7 cc./kg. intraperit.)								
No. rats	6		6		6		6	
Control	72.7±2.9		76.3±1.7		64.5±2.5		65.7±2.4	
1 hour	86.6±3.4		83.0±2.8		59.5±1.3		58.7±2.4	
2 hours	105.2±3.6		92.3±2.7		52.1±3.2		53.0±3.4	
3 hours	98.6±8.4		92.5±2.1		47.9±3.1		47.8±2.7	
4 hours	89.2±4.7		86.7±5.0		44.6±3.1		45.6±1.9	
5 hours	84.3±3.4		82.1±6.6		41.2±2.6		42.9±1.3	
6 hours	80.9±2.5		80.8±4.3		38.7±1.8		44.6±1.5	
D. Adrenalin (1 cc. 1:25,000/kg. intraperit.)								
No. rats	6	6	6	6				
Control	77.6±1.3	77.6±2.0[3]	78.1±1.4	76.7±1.6[1]				
½ hour	109.9±1.6	111.8±2.0[3]	109.5±2.9	110.1±2.2[1]				
1 hour	83.5±2.5	87.1±1.8[3]	83.5±2.7	88.3±2.0[1]				
No. rats		6						
Control		77.6±2.0[4]						
½ hour		129.3±3.7[4]						
1 hour		109.7±4.3[4]						
E. Insulin (0.01μ/100 gm. intraperit.)								
No. rats					6	6	6	
Control					66.8±1.4	67.3±1.2[5]	65.1±1.9	
1 hour					43.7±1.4	44.4±1.8[5]	44.5±1.9	
F. Thyroxin effect on reactivity of autonomic centers to metrazol (40 mg./kg. subcut.)								
No. rats	6	6						
Control	77.1±2.4	77.4±1.5[2]						
15 minutes	91.2±3.3	97.6±2.2[2]						
30 minutes	104.0±3.3	111.2±1.9[2]						
45 minutes	110.0±2.7	120.9±2.3[2]						
60 minutes	96.6±3.0	112.6±2.5[2]						
No. rats		6						
Control		78.3±1.4[4]						
15 minutes		100.1±2.0[4]						
30 minutes		120.0±2.8[4]						
45 minutes		132.6±3.9[4]						
60 minutes		122.4±3.8[4]						
G. Metrazol (40 mg./kg. subcut.)								
No. rats					6	6		
Control					67.3±1.6	67.3±1.6		
15 minutes					51.6±1.9	52.4±2.5		
30 minutes					48.5±1.5	48.1±1.7		
45 minutes					55.1±1.6	55.2±0.8		
60 minutes					59.2±1.2	62.2±1.5		

[1] Dosage of thyroxin=0.04 mg./100 gm./day for 5 days.
[2] Dosage of thyroxin=0.04 mg./100 gm./day for 6 days.
[3] Dosage of thyroxin=0.05 mg./100 gm./day for 4 days.
[4] Dosage of thyroxin=0.2 mg./100 gm./day for 6 days.
[5] Six doses of 0.2 mg. thyroxin/100 gm. for 10 days.

ments of table 1*A* but the differences are still more striking. It is seen that the hyperglycemia of the vagotomized-thyroidectomized rats is significantly less than that of the vagotomized animals. Here again it is found that no significant differences exist between the reaction of the adreno-demedullated and the adreno-demedullated-thyroidectomized rats.

The third series was performed by using typhoid-paratyphoid vaccine as a stimulus of the autonomic centers (table 1, C, fig. 3). The differences are less pronounced than in the other two series but it is found here again that the vaccine causes a lesser hyperglycemic response in the vagotomized-thyroidectomized rats than in the vagotomized controls. The significant difference occurs 2 hours after the administration of the vaccine. There are, however, no differences in the reactivity of the adreno-demedullated-thyroidectomized and the adreno-demedullated rats. These experiments show clearly that the removal of the thyroid influences the reactivity of the sympathetico-adrenal system without affecting that of the vago-insulin system.

The following experiments were designed to analyze the point of attack of these effects more closely. Table 1, *D*, shows effects of adrenalin on the blood sugar in vagotomized and in thyroidectomized-vagotomized rats. The results are conclusive in

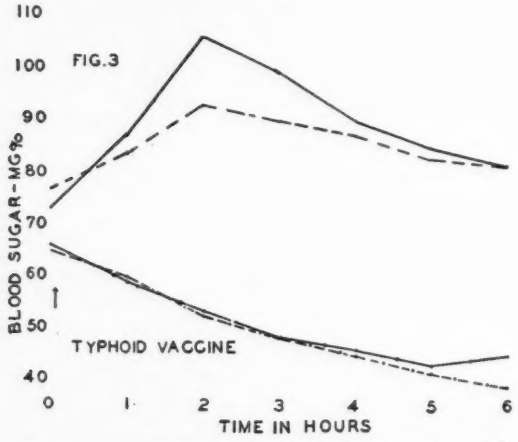

Fig. 3. Effect of typhoid-paratyphoid vaccine (0.7 cc./kg. intraperitoneally) on the blood sugar of the rat. (See fig. 1 for curve designations.)

showing that thyroidectomy does not alter the sensitivity of the blood sugar reaction to adrenalin. Table 1, *E*, shows similar experiments in which the effect of insulin was studied on the blood sugar in adreno-demedullated rats before and after removal of the thyroid. Here again no significant change in the hypoglycemic reaction was observed.

That the reactivity of the sympathetico-adrenal system is greatly decreased by thyroidectomy could be due either to the fact that the thyroid hormone increases in physiological quantities the excitability of the center regulating the secretion of adrenalin or to the potentiating influence of the thyroid hormone on the effectiveness of adrenalin on glycogenolysis in liver and muscles. Moreover these two effects could occur simultaneously. Since table 1, *D*, shows conclusively that thyroidectomy does not alter the effect of adrenalin on the blood sugar although it decreases the hyperglycemia resulting from procedures acting on the center of the sympathetico-adrenal system, it follows that those quantities of the thyroid hormone which are secreted under physiological conditions in the normal animal increase the excitability of the center of the sympathetico-adrenal system, but do not influence the blood sugar raising effect of adrenalin.

The observation that the effect of insulin on the blood sugar remains quantitatively unchanged by the removal of the thyroid and the further phenomenon that cen-

trally stimulating drugs such as metrazol, vaccine, etc., do not alter the reactivity of the vago-insulin system proves conclusively that the thyroid hormone in the amounts secreted by the normal gland is of no influence on the center of the vago-insulin system nor does it show any interaction with insulin which might take place after insulin has been secreted.

In the experiments involving the use of typhoid-paratyphoid vaccine not only the blood sugar but also the temperature reaction was studied. It has been found in an earlier investigation (4) that typhoid vaccine causes a rise in temperature in normal and vagotomized rats but a fall in temperature in adreno-demedullated animals. Since this fall is much greater than that found in adreno-demedullated-vagotomized animals, it is in part due to the secretion of insulin whereas the rise in temperature in normal

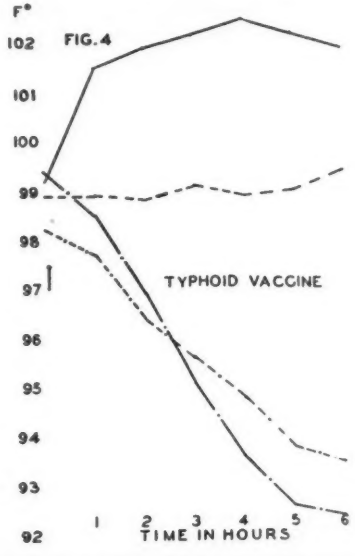

Fig. 4. Effect of typhoid-paratyphoid vaccine on the body temperature of the rat. (See fig. 1 for curve designations.)

and vagotomized animals must be attributed to the liberation of adrenalin. Figure 4 shows clearly that whereas vagotomized rats show a marked fever reaction the temperature remains unchanged in vagotomized-thyroidectomized rats after administration of the vaccine, but no significant differences in the reaction of the body temperature are observed between adreno-demedullated and adreno-demedullated-thyroidectomized rats. The temperature experiments confirm the conclusions drawn from the experiments described earlier inasmuch as these experiments again show the specificity of the thyroid hormone for the sympathetico-adrenal system. Since it is evident from our earlier work that the rise in temperature following the administration of typhoid-paratyphoid vaccine is due to the secretion of adrenalin, and since this effect is completely abolished by thyroidectomy it follows that the thyroid hormone in physiological concentrations increases the calorigenic effect of adrenalin; but no interaction occurs between thyroid hormone and insulin with regard to the regulation of the temperature although the insulin secretion controlled by the vagus lowers the

temperature of the body when the centers of the vago-insulin system are stimulated by typhoid-paratyphoid vaccine (4).

Effect of thyroxin on the vago-insulin and sympathetico-adrenal systems. Several series of experiments were performed in which vagotomized and adreno-demedullated rats were injected daily with thyroxin in order to study the effects of thyroxin on the vago-insulin and sympathetico-adrenal systems. It was found that the injection of 0.05 mg. thyroxin per 100 gm. body weight for 4 days increases distinctly the hyper-

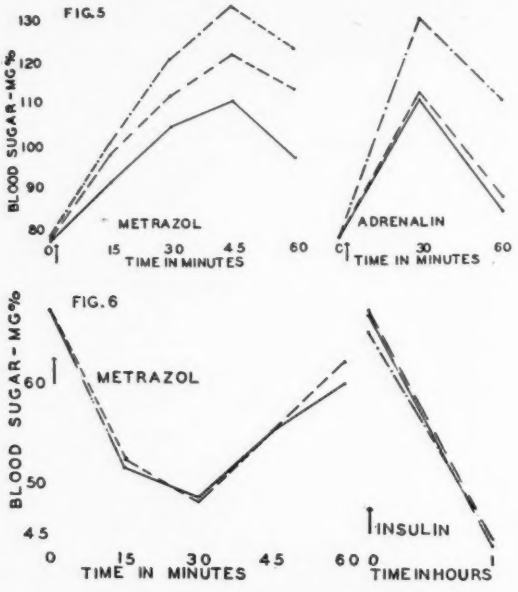

Fig. 5. Left side of figure, effect of metrazol (40 mg./kg. subcut.) on the blood sugar of the rat. Right side of figure, effect of adrenalin (1 cc. 1:25,000/kg. intraperitoneally) on the blood sugar of rats. [Solid line, vagotomized; dashes, vagotomized and on thyroxin (0.05 mg./100 gm. daily for 4 days); dashes and dots, vagotomized and on thyroxin (0.2 mg./100 gm. daily for 6 days.)] Fig. 6. Left side of figure, effect of metrazol (40 mg./kg. subcut.) on the blood sugar of the rat. Right side of figure, effect of insulin (0.01 u 100 gm. intraperitoneally) on the blood sugar of rats. [Solid line, adreno-demedullated; dashes, adreno-demedullated and on thyroxin (6 doses of 0.2 mg./100 gm. for 10 days); dashes and dots, adreno-demedullated-thyroidectomized.]

glycemic effect of metrazol on vagotomized rats (table 1, *F*, fig. 5). This difference is statistically significant. Since vagotomized rats subjected to this amount of thyroxin do not show any alteration in response to adrenalin (table 1, *D*, fig. 5) it follows that the increased hyperglycemic effect of metrazol is due solely to an increased excitability of the centers of the sympathetico-adrenal system. These conclusions were confirmed in another series of experiments in which thyroidectomized-vagotomized rats were injected with 0.1 mg. thyroxin per 250 gm. body weight, daily for 5 days (table 1, *A*). Under these conditions thyroxin offset not only the effects of thyroidectomy but also led to an increased reaction of the sympathetico-adrenal centers to metrazol as compared with that found in vagotomized control animals. In spite of this increase the hyperglycemic effect of adrenalin on the liver was not altered (table 1, *D*).

In another series of experiments the daily dosage of thyroxin was increased to 0.2

mg. per 100 gm. body weight and was administered for 6 days. If then the effect of metrazol was studied it was found that the hyperglycemic effect of metrazol was still greater than was observed in the preceding series (table 1, *F*, C, fig. 5). In order to decide whether this effect is due to an alteration in the excitability of the centers or to an interaction between thyroxin and adrenalin on peripheral structures or to both factors, the effect of adrenalin on these animals was again studied and compared with control animals not subjected to thyroxin. As table 1, *D*, and figure 5 shows this increased administration of thyroxin produced a very marked increase in the hyperglycemic effect of adrenalin. If one tries to get a quantitative expression by calculating the quotients of the blood sugars in the thyroxinized and in the control animals subjected to adrenalin and metrazol respectively, it is found that the increase in the hyperglycemic effect of adrenalin is similar to the increase in the hyperglycemic effect of metrazol. This seems to indicate that under conditions involving the administration of relatively large quantities of thyroxin which also lead to some pathological effects (weight loss), the alteration in the sympathetico-adrenal system is largely if not exclusively due to the interaction of thyroxin with that of adrenalin. On the other hand smaller doses of thyroxin may increase the excitability of the sympathetic centers without altering the effect of adrenalin on the liver.

In contradistinction to the effects of thyroxin on the sympathetico-adrenal system no effect on the vago-insulin system occurred in experiments in which thyroidectomized-adreno-demedullated and adreno-demedullated rats were subjected to metrazol before and after administration of thyroxin. In spite of the fact that the larger concentrations of thyroxin produced toxic effects, the reactivity of these animals to metrazol as well as their reaction to insulin remained unchanged (table 1 *A*, *G*; fig. 6).

DISCUSSION

The experiments have clearly shown by a comparison of the reactions studied in thyroidectomized and normal animals as well as by experiments involving moderate doses of thyroxin that, in physiological limits, the thyroid hormone increases the excitability of the centers of the sympathetico-adrenal system without influencing the glycogenolytic action of adrenalin. Only under conditions involving excessive amounts of thyroxin could it be found that there was a greatly increased glycogenolytic action of adrenalin on the liver which completely accounted for the increased effects of metrazol on the blood sugar of thyrotoxic animals. The vago-insulin system was not influenced either in its peripheral or in its central structures by the thyroid hormone. Thyroidectomy as well as thyroxin, even when this hormone was administered in toxic quantities did not alter the hypoglycemic response to metrazol and other stimuli acting on the autonomic centers.

In spite of the fact that thyroxin increases the metabolism of various tissues including those of the brain it acts specifically on the excitability of the sympathetic centers and does not influence the parasympathetic centers of the vago-insulin system. The cause of this specificity is unknown but worthy of note inasmuch as numerous drugs which were heretofore known as specific stimulants of the sympathetic and parasympathetic centers respectively, such as metrazol, cocaine, typhoid vaccine, bulbocapnine, picrotoxin, and others have been found to act on both sympathetico-adrenal and vago-insulin systems (Gellhorn and collaborators, 4). The fact that calcium or magnesium deficiency acts in a similar manner[3] suggests that specific ionic changes may result from the removal of the thyroid and from the administration of thyroxin. It is not improbable that the accumulation of iodine in the midbrain which

[3] Unpublished observations.

is produced by the injection of thyroxin (5) is responsible for the specific effects on the center of the sympathetico-adrenal system.

It has been stated also that under the conditions of thyrotoxicosis the glycogenolytic action of adrenalin is greatly increased in our animals. This is analogous to the increased excitatory effects obtained with adrenalin on peripheral sympathetic structures in thyrotoxic animals (6). It is interesting that in thyrotoxicosis the effect of adrenalin is increased not only on sympathetic effectors, e.g., in the heart, but also on the liver which is the effector organ of the sympathetico-adrenal system as far as the blood sugar is concerned although the liver seems to be devoid of sympathetic nerve fibers (7, 8).

SUMMARY

The effect of thyroidectomy and of thyroxin on the vago-insulin and sympathetico-adrenal systems is studied. The former reacts on central stimulation with hypoglycemia in adreno-demedullated rats, the latter responds with hyperglycemia in vagotomized animals. It is found that metrazol, electrically induced convulsions, and typhoid-paratyphoid vaccine, produce a greater hyperglycemic effect in vagotomized rats than is found in thyroidectomized-vagotomized rats. Since no difference in the effect of adrenalin on the blood sugar is observed in the two groups, it is concluded that the thyroid hormone in physiological quantities increases the excitability of the center controlling the sympathetico-adrenal system without influencing the reactivity of the liver to adrenalin. Conversely it is observed that the hyperglycemic effect of metrazol is increased by administration of small doses of thyroxin. Under these conditions the effect is also due to an increased central excitability and not to sensitization of the liver to the effect of adrenalin. If, however, thyroxin is administered in toxic quantities the hyperglycemic effect of adrenalin is increased to such a degree that it completely accounts for the increased hyperglycemia produced by metrazol and other procedures acting on the centers of the sympathetico-adrenal system.

The fever produced by typhoid-paratyphoid vaccine in vagotomized animals is abolished by thyroidectomy.

The vago-insulin system is in its central as well as in its peripheral parts uninfluenced by either the removal of the thyroid or by the addition of physiological and toxic concentrations of thyroxin.

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EFFECTS OF HORMONES ON THE SKELETON OF MICE, GUINEA PIGS AND RATS[1]

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DURING the processes of skeletal growth and ageing, three main phases may be distinguished: (*a*) During the first period proliferation of the epiphyseal cartilage predominates, producing lengthwise growth of the long bones. Towards the end of this period proliferative processes decline and the epiphyseal disks narrow gradually. Simultaneous periosteal growth processes cause an increase in thickness of the bone. (*b*) During the second period retrogressive changes, such as atrophy, disintegration, hyalinization and calcification of the epiphyseal cartilage predominate. These changes, associated with or followed by ossification, coincide with the cessation of the lengthwise growth of the long bones and with a further increase in the density and thickness of the bony shaft. (*c*) During the third period, ossification of the epiphyseal cartilage progresses, but at the same time processes of resorption of the bone by elements of the bone marrow and of the periosteal tissue begin to predominate. This leads to a thinning and to more or less extensive perforations of the epiphyseal disks and to a thinning of the shaft of the long bones. These three periods may to a certain extent overlap, and their duration varies greatly in different strains of mice. In strain C_{57}, which in our studies served as standard and which exhibits a comparatively slow rate of skeletal ageing, the first period lasted until about the end of the fourth month of life, the second until about the end of the first year, when the third and final phase set in (1).

In previous investigations we were able to show that the course of the periods of bone growth can be altered under experimental conditions. The purpose of the present report is to compare some of the changes in cartilage and bone called forth by these various procedures.

Influence of Anterior Pituitary Preparations on Cartilage and Bone of Growing Animals

(*a*) Injections of *bovine anterior pituitary extract* (2) caused a transitory stimulation of the proliferation and hypertrophy of the epiphyseal and articular cartilage, and an increased mitotic proliferation of osteoblasts in the metaphysis. The maximum of these changes was reached after about 2 weeks of treatment; following this interval, the rate of proliferation dropped sharply, even below normal. Thus, although the proliferation was temporarily intensified, its actual duration was shorter than normal. The subsequent period, that of predominance of retrogression and ossification of cartilage, set in at an earlier age than usual, and these effects were also more marked. The epiphyseal disks underwent, therefore, premature closure, the bony spicules

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and the cortex of the shaft were temporarily thickened, and the incidence and severity of arthropathic lesions in the joints were increased. The third period, that of predominance of resorptive processes, likewise began earlier than usual and these changes were intensified, as indicated by an early appearance of perforations of the epiphyseal disks and a still greater increase in the arthropathic lesions; eventually, an advanced degree of epiphyseo-diaphyseal union was produced and the thinning of the shaft of the long bones made rapid progress.

Similar but more marked changes were observed in growing guinea pigs under the influence of anterior pituitary extract (3). Stimulation of proliferation of cartilage subsequent to the administration of anterior pituitary preparations has been reported in rats (4, 5) in guinea pigs (6), and in dogs (7), and Handelsman and Gordon (8) described an increase of bone production in young rats.

(*b*) *Anterior pituitary 'growth hormone'*[2] injected subcutaneously caused, in growing mice and immature guinea pigs, changes similar to those called forth by the bovine anterior pituitary extract prepared in this laboratory. The proliferation of cartilage was temporarily increased and then it ceased prematurely. The processes predominating during the second period, namely those of degeneration of cartilage and deposition of bone, likewise set in earlier and proceeded more rapidly and more intensely than usual, as did the processes of resorption during the third period. However, the changes produced by injections of the same volume of anterior pituitary 'growth hormone' were less pronounced than those induced by anterior pituitary extract of cattle. According to Ross and McLean (9), in 6-month-old rats, intraperitoneal injections of anterior pituitary growth hormone were ineffective but stimulation of growth of cartilage was seen if the preparation was given subcutaneously.

(*c*) *Implants of bovine anterior pituitary* in immature guinea pigs (10) caused likewise a stimulation of the proliferation of the epiphyseal and articular cartilage, and premature appearance of degenerative processes. However, owing to the fact that such heterotransplants remain alive and active for only a short time, there were no later effects of such implants to be observed.

(*d*) *Syngenesiotransplants of the anterior pituitary* in closely inbred strains of mice (2) induced an initial inhibition of the proliferation of the epiphyseal cartilage, but the subsequent period during which proliferation took place lasted longer than normal; in other words, the cartilage continued to proliferate, although at a slow rate, at an age when normally this process would have ceased and degenerative changes have become predominant. More bone was present in the metaphysis and in the shaft than either normally or after administration of anterior pituitary extracts. The second period, characterized by retrogressive changes and deposition of bone, set in later than usual; but once it had begun, it progressed rapidly and with greater intensity than ordinarily. The third phase, with predominance of resorptive processes, began earlier, the epiphyseo-diaphyseal union was accelerated and the degree reached was more advanced than in normal animals, but less than after treatment with anterior hypophyseal extracts.

Thus, both the anterior pituitary extracts and transplants of anterior hypophysis caused, in the end, an acceleration of skeletal ageing, but the underlying mechanism was different in these cases. Whereas, under the influence of anterior pituitary extracts the proliferation of the cartilage was stimulated for a short time and the proliferative phase shortened, under the influence of the transplants a temporary inhibition of the proliferation of the cartilage and a prolongation of the proliferative period occurred. The processes of resorption were more pronounced by the action of extracts, the formation of bone more accentuated by the action of transplants.

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[2] Antuitrin-G, Parke, Davis and Co., Detroit, Michigan.

Influence of Thyroid Hormone on Cartilage and Bone of Growing Animals

In mice, subcutaneous injections of thyroxin (11) caused at first a marked stimulation of the proliferation and hypertrophy of the epiphyseal cartilage, which was stronger than that observed after administration of anterior pituitary extracts. After this first period there was a sharp decline in the rate of proliferation, followed by a premature onset and intensification not only of the processes of degeneration and ossification, but also of resorption of cartilage and bone. The processes of resorption were more striking than in the case of either anterior hypophyseal extracts or transplants. They were so markedly accelerated that perforations in the epiphyseal disks appeared at a time when ordinarily proliferation of epiphyseal cartilage is still in progress; furthermore, these processes were so greatly intensified that, at later stages, complete epiphyseo-diaphyseal union occurred in mice. We did not observe a similar degree of epiphyseo-diaphyseal union in old mice under normal conditions, and only rarely after administration of anterior pituitary hormone. The compacta of the shaft was much thinner in mice injected with thyroxin than in normal old mice or after administration of anterior pituitary hormone.

Feeding of thyroid tablets to guinea pigs (12) led to similar but less marked changes.

In dogs (7), guinea pigs (13), and rats (14), thyroid hormone likewise caused premature closing or perforations of the growth zones and increased processes of absorption. On the other hand, removal of the thyroid gland in growing guinea pigs (15) did not induce a cessation of the proliferation of cartilage, but the conversion of the columnar into hypertrophic cells, as well as calcification and subsequent ossification of the latter, was greatly decreased. The resorptive processes were likewise distinctly diminished.

Influence of Potassium Iodide on Cartilage and Bone of Growing Animals

Intraperitoneal injections of potassium iodide in guinea pigs (16) and mice led to changes comparable in kind to those noted after administration of thyroxin, but they were less in degree. The increase in the proliferation of cartilage was less accentuated after injections of potassium iodide than after injections of thyroxin; the retrogressive changes appeared earlier than normal but later than after treatment with thyroxin, as was noted also in the onset and progress of the resorptive processes. The thinning of the bony shaft and the degree of epiphyseo-diaphyseal union reached after prolonged injections of potassium iodide were far less advanced than after thyroxin and also less than after administration of anterior pituitary hormone.

These effects on the epiphyseal cartilage could be observed also in growing guinea pigs in which the thyroid glands had been removed (17) previous to the administration of potassium iodide, and it is, therefore, probable that potassium iodide acts to a large extent directly on the skeletal tissues and without the intermediation of the thyroid gland. However, in these thyroidectomized animals the resorption of cartilage and bone lagged behind that seen in normal animals as well as in those treated with potassium iodide but possessing intact thyroid glands. It is therefore likely that the increase of resorptive processes observed after administration of potassium iodide may be at least partly mediated by the thyroid gland.

Influence of Parathyroid Extract and Calcium Gluconate on Cartilage and Bone of Growing Animals

Intraperitoneal injections of 4 U of parathyroid extract[3] 3 times weekly, caused an increased calcification, degeneration and ossification of the epiphyseal cartilage,

[3] Eli Lilly and Company.

but there was no stimulation of its proliferation in 5 weeks old mice kept on a standard diet, at a time when under normal conditions the proliferation of the epiphyseal cartilage is still quite marked. But there was an accumulation and an accentuation of mitotic proliferation of osteoblasts in the subepiphyseal layer, leading to a temporary increase in the number and thickness of the trabeculae, as was found also by Bauer, Aub and Albright (18), Selye (19), as well as by Shelling, Asher and Jackson (20) in rats. With a longer continued administration of the parathyroid extract the processes of calcification, degeneration and ossification made further progress, but they were then associated also with an intensification of processes of resorption, causing in 10 weeks old mice circumscribed perforations of the epiphyseal plate and a thinning of the shaft. In the metaphyseal tissue the excess bone was resorbed and a fibrillar connective tissue developed between the spicules. But these changes remained restricted to the subepiphyseal zone and no severe lesions indicative of fibrous osteitis were observed. In puppies, guinea pigs (21) and rats (22) changes characteristic of fibrous osteitis were produced subsequent to the administration of parathyroid hormone. However, only a slight increase of resorptive processes was observed under prolonged treatment of rabbits (23). After administration of parathyroid extract Bauer, Aub and Albright (18) obtained negative results in cats, whereas in rabbits resorption of bone predominated. Burrows (24), on the other hand, observed in cats a primary increase in the absorption of bone, followed by an over-production of bone and a final return to a more normal condition. It is probable that differences in the species used, in the duration of the experiment, in the age of the animal, and in the dosage of the hormone play a determining role in the response of the skeletal tissue to parathyroid extract.

According to the histological findings in mice, parathyroid extract promotes the ageing processes in cartilage and bone similar to the action of thyroxin. In contradistinction to the action of thyroxin, in mice kept on a standard diet parathyroid extract does not stimulate the proliferation of the cartilage.

The early changes observed following intraperitoneal injections of 0.25 cc. of 10% calcium gluconate[4] consisted in an increased degeneration, calcification and ossification of the epiphyseal cartilage, and in an intensified bone formation in the spicules and the shaft. Bauer (18) and his collaborators have recorded a similar increase of bone formation in cats and rabbits under the influence of a diet high in calcium. In mice with longer continued administration of calcium gluconate we found no further progress in these old age changes in cartilage and bone, but a calcareous incrustation of the testicles and deposits of calcium on the surface of liver and spleen were noted at autopsy.

The combined administration of calcium gluconate and parathyroid hormone induced in cartilage and bone no intensification of the old age changes seen after injections of parathyroid hormone alone.

Influence of Sex Hormones on Cartilage and Bone of Growing Animals

(*a*) *Estrogen.* In guinea pigs the first changes consisted in a marked narrowing of the epiphyseal disks caused by an inhibition of proliferation and an increased calcification and hyalinization of the epiphyseal cartilage (25). Whereas, in immature guinea pigs under the influence of moderate doses of this hormone the suppression of growth of cartilage was only transitory and was followed by an enlargement of the epiphyseal plates associated with an increased proliferation of cartilage cells, in growing mice and in some rats we could not observe such a stimulation of the proliferation of

[4] Abbott Laboratories.

cartilage, but the phase of inhibition was soon followed by accelerated and intensified processes of retrogression and ossification (26). However, resorption of cartilage and bone was at first greatly inhibited. Thus, the breakdown of the epiphyseal cartilage was delayed, the subepiphyseal trabeculae persisted for an abnormally long time and the shaft was thickened. These findings correspond to those of Day and Follis (27) in growing rats. The amount of bone in the diaphysis of mice was further increased by apposition of new bone. This was, however, not accomplished by increased activity of osteoblasts undergoing mitotic division, as it was in the case of anterior hypophyseal extracts, but by a coalescence of connective tissue or bone marrow cells, which, in contact with the pre-existing bone, deposited osseous substance. These processes led to the formation of an interlaced osseous network which encroached upon the marrow cavity in the direction from the subepiphyseal layer towards the diaphysis; this increased bone formation involved also the shaft and at the time of the maximal effect could lead to the occlusion of a great part of the marrow cavity. Similar observations were reported in rats and in chickens (28), in mice (29, 30), and in pigeons sparrows and ducks (29). The extent of these changes varied in different strains of mice and in different species (26), some of the rats and all of the guinea pigs showing only very little tendency to increased deposition of bone. Moreover, in the response of avian bones to the administration of estrogen, seasonal conditions were noted (31), hyperossification occurring in pigeons injected in the spring but not in those injected in December. In our experiments, notwithstanding the continued administration of estrogen, resorptive processes were resumed in mice, and these brought about a removal of the excess bone as well as a belated perforation of the epiphyseal disks. In rats, Bremer (32) observed increased absorption of bone after administration of estrogen, the changes resembling those seen in osteitis fibrosa.

(*b*) *Testosterone.* Injections of testosterone (33) caused at first a temporary inhibition of the proliferation of the cartilage and of the absorption of cartilage and bone, effects similar in kind but less in degree than those seen after administration of estrogen; they led to a prolonged persistence of the metaphyseal spicules and to a slight thickening of the cortex of the shafts. Subsequently, the onset and progress of retrogressive changes and of ossification of the cartilage were accelerated and intensified as compared with these processes in the normal animal, but to a lesser degree than in the case of estrogen. Simultaneously, the apposition of bone was slightly increased and, in the metaphysis, a loosely interlaced trabecular network was formed, which extended, however, not as deeply into the marrow cavity as after administration of estrogen. As in the latter case, but at an earlier stage of the experiment, processes of resorption were resumed and proceeded with increased intensity. The excessive amount of bone formed previously was resorbed and perforations of the epiphyseal disks could be initiated even at an earlier date than normally.

(*c*) *Progesterone.* After administration of progesterone (34), the proliferation of the epiphyseal and articular cartilage was unchanged or slightly stimulated. The onset of degenerative changes in the cartilage was delayed and ossification lagged behind the normal rate. Thus the youthful character of the cartilage was maintained for a period longer than usual. This effect represents, therefore, to a certain extent, the opposite of the one exerted by estrogen and testosterone, both of which accelerate the ageing of the epiphyseal cartilage. Moreover, the osseous substance deposited at the shaft and the subepiphyseal spicules contained a relatively large number of cells and little ground substance, and thus likewise retained a youthful character for an increased length of time. At later stages, however, the processes of retrogression, ossification and resorption progressed at a faster rate than ordinarily, thus equalizing the delay experienced during earlier stages of the treatment. After prolonged administration

of progesterone, the degree of epiphyseo-diaphyseal union and the thickness of the osseous shafts were therefore comparable to those seen in untreated mice of corresponding age.

(*d*) *Gonadectomy* produced changes in cartilage and bone (35) which were not unlike those taking place after the administration of progesterone; but, whereas after injections of the latter substance the stimulation of proliferation of the epiphyseal and articular cartilage was slight at best, it was fairly strong subsequent to gonadectomy. Similarly, as in the case of progesterone, the processes of degeneration and ossification were delayed after gonadectomy. The epiphyseal disks were temporarily enlarged, the cartilage cells were better preserved than in normal animals of corresponding age, while degenerative processes were decreased and retarded. Less bony substance was deposited than usual in the metaphysis and in the shaft. But in contrast to conditions seen in animals treated with progesterone, the processes of resorption in the bony structures were fairly active. In old gonadectomized animals the epiphyseal disks still contained more cells than usual, but the processes of degeneration, and particularly those of resorption, had made fast progress and so had led to the production of a degree of epiphyseo-diaphyseal union which was advanced beyond that seen after administration of progesterone or in normal old animals.

DISCUSSION

In regard to the ageing process, Loeb (36) concluded that each organ has its own peculiar time curve, which later ends in the phase of senescence and finally in death. These characteristics of the time curve are fixed by heredity, but they can be modified within certain ranges by environmental factors, and in particular also by hormones. These may either accelerate or retard, weaken or intensify, certain phases of the time curve, without inducing profound alterations. Different hormones may differ in the way in which they affect different parts of the time curve; they may accelerate one and retard another, and various hormones may differ in these respects. These differences apply to different species and even to different strains. With these views our observations on the effects of hormones on the skeletal system of mouse, rat and guinea pig are in agreement.

We may state that some hormones or hormone-like substances affect the processes of growth and ageing of the skeletal tissues of growing animals in a similar manner. On this basis, anterior hypophyseal extracts, thyroid hormone, and potassium iodide may be grouped together. The effect of these substances is characterized by an acceleration or intensification of all three phases of skeletal growth and ageing, this acceleration being strongest in the case of thyroid hormone, next strongest in the case of anterior pituitary extracts, and weakest in the case of potassium iodide. Proliferation as well as degeneration and ossification of cartilage, and resorptive processes in both cartilage and bone proceed at an accelerated rate, and these substances cause, therefore, premature skeletal ageing.

Parathyroid hormone and, to a lesser extent, calcium gluconate, accelerate skeletal ageing by promoting calcification and degeneration of the cartilage, and by increasing the processes of ossification and of absorption of cartilage and bone. But these substances do not intensify the proliferation of the cartilage.

Estrogen and testosterone, on the other hand, are similar in their effects on the growing skeleton, in that they inhibit proliferation and hypertrophy of the growing cartilage, and also, at least temporarily, resorptive processes in cartilage and bone, while inducing at the same time increased degeneration of the cartilage, estrogen being more effective in this respect than testosterone. Thus, these two hormones resemble those of the first group insofar as they accelerate ageing of the skeleton, but they differ

from the latter in that they fail to induce a primary proliferation of cartilage and delay resorption of cartilage and bone while increasing bone formation.

In a certain way, syngenesiotransplants of the anterior hypophysis in mice took an intermediate position between *groups* 1 and 3. While in the later stages age changes were likewise accelerated and conditions in this respect were very similar to those exerted by the substances mentioned in the first group, there was noticeable a primary inhibition of the proliferation of cartilage, similar to that caused by the third group; but they differed from the latter in that this inhibition was subsequently compensated by a prolongation of the growth period.

Gonadectomy produces a fourth group of effects opposite to the aforementioned ones, as does also the administration of progesterone. Both of these factors induce a primary delay of the onset and progress of skeletal ageing, but the resulting retardation of skeletal ageing is equalized at later stages by an intensification chiefly of the processes of resorption.

The action of these different hormones on cartilage and bone varies in degree, not only in different species but even in different strains of the same species. It could be shown that representatives of those strains which normally exhibit a fast rate of skeletal ageing (D mice) are less responsive to the hormones than animals of strains with a slow rate (C_{57} and CBA mice). This difference may be correlated with the observation that, in a general way, the effects of hormones on the adult and aged skeleton are less pronounced than on the growing skeleton (37). While some of these investigations are still in progress, it may be stated that there is as yet no evidence that cartilage which has completely ceased to proliferate and is in a more or less advanced stage of degeneration and ossification at the beginning of the treatment, can be induced to resume its growth. On the other hand, these investigations indicate that such processes as are in progress in the skeletal tissues in earlier life, may be accelerated and intensified or they may be retarded and weakened.

SUMMARY

Hormones or homone-like substances may accelerate and intensify, or retard and weaken the processes of skeletal growth and ageing. Anterior hypophyseal extracts, potassium iodide, parathyroid and thyroid hormones, estrogen and testosterone accelerate and intensify the processes of skeletal ageing. The first three substances cause a temporary stimulation of the proliferation of cartilage in growing animals, associated with a shortening of the growth period, whereas estrogen and testosterone inhibit the proliferation of cartilage. Gonadectomy and progesterone inhibit temporarily the processes of skeletal ageing. The effects of these different hormones on cartilage and bone vary in various species, in various strains in the same species, and according to the age of the animal used.

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